ANTIBIOTIC RESISTANCE IN *NEISSERIA GONORRHOEAE*: IMPACT OF

CEFTRIAXONE RESISTANCE ON MICROBIAL FITNESS AND POTENTIAL OF

RESISTANCE DETERMINANTS TO SPREAD DURING MIXED INFECTION

by

Leah Rebecca Vincent

Dissertation submitted to the Faculty of the
Emerging Infectious Diseases Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2016

FINAL EXAMINATION/PRIVATE DEFENSE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN THE EMERGING INFECTIOUS DISEASES GRADUATE PROGRAM

Name of Student: Leah Vincent

Date of Examination: March 7, 2016
Time: 1:30 PM
Place: B3004

DECISION OF EXAMINATION COMMITTEE MEMBERS:

	PASS	FAIL
DS Merrell	X	__
Dr. D. Scott Merrell DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY Committee Chairperson		
Ann Jerse	X	__
Dr. Ann Jerse DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY Dissertation Advisor		
Alison O'Brien	X	__
Dr. Alison O'Brien DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY Committee Member		
Robert Nicholas	X	__
Dr. Robert Nicholas UNIVERSITY OF NORTH CAROLINA, CHAPEL HILL Committee Member		

APPROVAL OF THE DOCTORAL DISSERTATION IN THE EMERGING INFECTIOUS DISEASES GRADUATE PROGRAM

Title of Dissertation: "Antibiotic Resistance in Neisseria Gonorrhoeae: Impact of Ceftriaxone Resistance on Microbial Fitness and Potential of Resistance Determinants to Spread During Mixed Infection"

Name of Candidate: Leah Vincent
Doctor of Philosophy Degree
March 7, 2016

DISSERTATION AND ABSTRACT APPROVED:

DATE:

Dr. D. Scott Merrell
DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
Committee Chairperson

3/7/16

Dr. Ann Jerse
DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
Dissertation Advisor

3/18/16

Dr. Alison O'Brien
DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
Committee Member

3/7/16

Dr. Robert Nicholas
UNIVERSITY OF NORTH CAROLINA, CHAPEL HILL
Committee Member

3/7/16

ACKNOWLEDGMENTS

COPYRIGHT STATEMENT

The author hereby certifies that the use of any copyrighted material in the dissertation manuscript entitled: Antibiotic Resistance in *Neisseria gonorrhoeae:* Impact of Ceftriaxone Resistance on Microbial Fitness and Potential of Resistance Determinants to Spread during Mixed Infection is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.
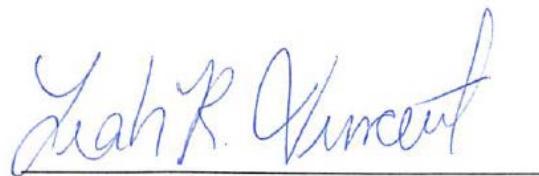
Leah R. Vincent
Emerging Infectious Disease, Microbiology Department
Uniformed Services University
March 18, 2016

DISCLAIMER

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the Department of Defense or the U.S. Government.

ABSTRACT

Antibiotic Resistance in *Neisseria gonorrhoeae:* Impact of Ceftriaxone Resistance on Microbial Fitness and Potential of Resistance Determinants to Spread during Mixed Infection:

Leah R. Vincent, Doctor of Philosophy, 2016

Thesis directed by: Dr. Ann E. Jerse, Professor, Department of Microbiology and Immunology

Approximately 106 million cases of gonorrhea occur worldwide each year, and antibiotic treatment is critical for control. However, the rapid evolution of drug resistance in *Neisseria gonorrhoeae* (Gc) threatens this strategy. Extended-spectrum cephalosporins (ESC), including ceftriaxone (Cro), are the last remaining monotherapy for gonorrhea. Increasing resistance of Gc to the ESCs has amplified the threat of untreatable gonorrhea. Cro resistance in Gc is conferred primarily by mosaic *penA* alleles that encode multiple amino acid substitutions within penicillin-binding protein 2 (PBP 2).

Here we examined the impact of mosaic *penA* alleles from Cro[R] strains H041 and F89 on Gc fitness *in vitro* and *in vivo*. We hypothesized that mosaic *penA* alleles that confer high-level ESC resistance (*penA41, penA89*) will confer a fitness cost *in vitro* and *in vivo* and that compensatory mutations may be selected that restore fitness without reducing antibiotic resistance. We also tested whether the *penA* allele could be transferred between two Gc strains during co-culture and in experimental mixed infection.

FA19 *penA41* and FA19 *penA89* grew significantly slower than strain FA19 *rpsL* and were out-competed by the Cro^S parent strain when co-cultured in broth. Strain FA19 *rpsL* also out-competed the Cro^R mutants during competitive genital tract infection in BALB/c mice; however, four Cro^R compensatory mutants (LV41A, LV41B, LV41C and LV41E) of strain FA19 *penA41* were isolated from several mice. High-throughput sequencing was used to identify differences among the mutant and parent strains.

We characterized the novel putative compensatory mutation in *acnB* (G348D), identified in strain LV41C. The mutation appears to increase fitness despite the fact that AcnB is not functional for metabolism in the mutant strain, which may reveal a potential new role for AcnB during gonococcal infection that cannot be detected under standard growth conditions but is detected *in vivo.* In addition to the *acnB*$_{G348D}$ mutation, a codon deletion was identified in *mleN* (ΔA467) in strain LV41A and LV41B. The enzymes encoded by these two genes are critical to metabolic processes. We conclude that compensatory mutations can be selected during infection with Cro^R Gc that increase fitness. This may facilitate the persistence and spread of Cro^R Gc strains. Furthermore, this work has opened new avenues of inquiry into Gc metabolism and physiology, and how changes in metabolic processing or physiology may alter Gc adaptation to the host environment.

Additionally, we demonstrated proof-of-concept that antibiotic resistance determinants can be transferred between pathogenic strains of Gc via natural transformation *in vitro.* We examined the *in vitro* and *in vivo* transfer of *penA41* and commonly isolated resistance alleles that confer intermediate level resistance to ciprofloxacin (*gyrA*$_{91/95}$) and macrolides (*mtrR*$_{-79}$). We hypothesized that each allele

would be transferred efficiently by natural transformation *in vitro*. We further

hypothesized that *in vivo* selection of the *penA41* allele would be rare due to the fitness

defect it confers, while selection of both the $gyrA_{91/95}$ and $mtrR_{-79}$ would be more frequent

due to the observed fitness benefit the latter two confer *in vivo*. We used strain OP100, an

FA1090 variant that carries chloramphenicol resistance as the recipient strain and created

recombinant strains FA19 *penA41*, *recA-*, AK1 *recA-,* and KH15 *recA-*, recombination-

deficient donor strains for transfer of resistance determinants. An *in vitro* co-culture

system was developed to identify transformants of the recipient strain that acquired the

desired resistance determinant via natural transformation. However, we were unable to

transfer the resistance alleles *in vivo* perhaps due to loss of piliation of the recipient strain

or a previously unrecognized fitness difference between the backgrounds strains FA19

(donor) and FA1090 (recipient).

  In vivo transfer of these resistance determinants may show how resistant strains

are able to disseminate internationally, with a particular emphasis on the relationship

between the fitness advantage associated with particular alleles and the frequency of

successful transfer. Further refinement of a coinfection model in mice is needed to test

this question thoroughly *in vivo*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: Introduction

1.1 THE GONOCOCCUS

The exclusively human pathogen *Neisseria gonorrhoeae* (Gc) is a Gram-negative diplococcus and is the etiological agent of the sexually transmitted infection gonorrhea. This bacterium has co-evolved with humans; indeed the first written records of gonorrhea date back to biblical times and are referenced in the Book of Leviticus within the laws of bodily discharge (Lev. 15:1-15:33) (43). The association is so close that it is now known that, unlike *Neisseria meningitidis* or commensal *Neisseria* species, 11% of Gc strains carry fragments of human DNA (8).

Gc is a fastidious organism with specific growth requirements for laboratory culture. It is microaerophilic, requiring 3%-5% CO_2, and only grows between 35-37 degrees Celsius under humid conditions. Gc can grow anaerobically in the presence of nitrite as a terminal electron acceptor, and anaerobic growth rates are equal to those observed during aerobic growth (82). Gc growth is improved on solid media with the inclusion of co-carboxylase and glutamine (85). In 1944, Gould *et al.* demonstrated that Gc required glutamic acid, histidine, glucose, starch, glutathione, sodium chloride, iron salts, magnesium, and phosphates (56). Interestingly, select samples of agar were inhibitory for growth, but the starch was shown to counteract the inhibitory effect of the agar (56). Chocolate agar was eventually used to isolate Gc. In clinical laboratory diagnosis, Gc is frequently cultured on modified Thayer-Martin chocolate agar, which is chocolate agar containing antibiotics that inhibit commensal flora (45).

The Gc genome is 2.15 Mb in length (60). Eighty percent of strains also contain a genetic island (122). Gc is naturally competent and uses a 10-bp DNA uptake sequence (DUS) that is specific to *Neisseria* species. Approximately 1,965 copies of this sequence are present in the Gc genome, and natural transformation is a major source of genetic diversity (58; 60). Unlike many other naturally competent bacterial species, Gc does not regulate competence, which allows transformation to occur at any stage of growth (58). Type IV pili are also required for natural competence; *in vitro* studies have shown that Type IV pili are also a mechanism of interspecies horizontal genetic transfer, specifically between Gc and the commensal *Neisseria elongata* (63). Plasmids are extragenetic elements that can serve as a vital means of genetic exchange. Ninety-six percent of clinical Gc isolates carry a 2.6 mega Dalton (MDa) cryptic plasmid, but this plasmid is not associated with any known virulence factors and has unknown function(s) (125). Although its purpose remains unclear, the cryptic plasmid most likely has a function since it is maintained in so many strains. This theory is supported by the fact that portions of the plasmid, both large and small, are incorporated into the chromosome, enhancing genetic diversity (125). Other plasmids carried by Gc strains are associated with antibiotic resistance. β-lactamase-encoding plasmids were originally associated with strains from specific geographic regions. However, these plasmids have spread from Africa and East Asia worldwide and are now ubiquitous throughout circulating strains of Gc (125). These plasmids were an important reservoir for the spread of non-chromosomal penicillin resistance, and their emergence was one step towards the inevitable end of penicillin as an effective treatment for Gc infection (87). Additionally, Gc strains can carry conjugative plasmids that aid in the spread of some β-lactamase plasmids. One of

these, the 25.2 MDa TetM plasmid, also confers resistance to tetracycline (125). Therefore, conjugation as well as natural transformation is a rich source of genetic diversity for Gc.

1.2 GONOCOCCAL INFECTION

The gonococcus gains entry to its host through sexual transmission, most commonly through the genital tract. There, it infects the mucosal surface of the urethra or cervix (110; 168). Less common sites of infection through sexual transmission include the pharynx and rectal mucosa. Additionally, infection of the conjunctivae by perinatal transmission can also occur (110; 168). Characteristics of symptomatic infection include an intense mucopurulent discharge that is the result of the strong localized inflammatory response at the site of infection. However, asymptomatic genital tract infections are also common. This is true particularly in women. Furthermore, rectal and pharyngeal infections are typically asymptomatic and thus serve as a reservoir of infection. In contrast, conjunctival infections are usually symptomatic and can lead to scarring and blindness (43; 167).

The infectious process is slightly more straightforward in men than in women. Typically, urethral infection in men is characterized by a pyogenic mucopurulent discharge. In untreated infections, the bacterium can ascend to the prostate or epididymis (75; 109). When treated, the infection clears in about 14 days in men. It is the columnar epithelium that lines the interior of the urethra that is susceptible to Gc infection (110). The spectrum of disease in men can range from asymptomatic to acute urethritis, acute prostatitis, or acute epididymitis. (75; 110).

Infection can occur at multiple sites in the female lower genital tract, comprising the cervix, urethra, Skene's or Bartholin's glands, or both (168). Disturbingly, forty-five percent of infections ascend to the upper genital tract and uterine tubes, as well as the abdominal cavity (43; 168). The disease can be particularly tragic in women, where asymptomatic infection combined with ascendant infection can lead to serious sequelae if left untreated. Between 10%-20% of women with undiagnosed or inadequately treated cervicitis, develop pelvic inflammatory disease (PID) that is associated with either gonorrhea or chlamydia. PID can result in chronic pelvic pain, scarring of the uterine tubes, and infertility, which are all lifelong consequences of infection.

Gc in men and women can also infect the pharynx, and infection in pregnant women can affect the conjunctivae of newborn infants, particularly in the developing world (47; 48; 168). Additionally, disseminated gonococcal infection (DGI), which can occur in up to three percent of infected persons, can lead to joint infection, septic arthritis and dermatitis. DGI occurs more frequently in young women (80; 168).

1.3 PATHOGENESIS

The need to colonize its human environmental niche drives the pathoadaptation of Gc. During colonization of nonsterile body sites, Gc must successfully compete with commensal organisms at the site of infection for nutrients while evading host defense mechanisms. This delicate interplay contributes to the virulence of Gc (97). Pathoadaptation includes, but is not limited to, virulence factors that overcome or evade host responses to infection, genetic mechanisms for responding to environmental stimuli and host immune factors, and specific metabolic pathways to meet nutritional needs while growing in the hostile host environment.

1.3a Virulence Factors

1.3a1 Adherence Factors

Initial attachment of Gc to epithelial cells of mucosal surfaces occurs through the Type IV pilus (43). Interactions between the host cell and the Gc pilus stimulate signal transduction pathways that may induce host cell cytokine responses and Gc invasion of cells (43; 46; 57; 173). Gc is a non-motile organism. However, studies have shown that Type IV pilus retraction, or twitching motility, allows micro colonies to form during infection and may be a mechanism to facilitate localized spread and ascent into the upper genital tract (43; 63).

The opacity (Opa) proteins mediate Gc invasion of host cells through interactions with either members of the carcinoembryonic antigen-related family of cell-adhesion molecules (CEACAMs), or heparin sulfate proteoglycans (HSPG) (132). Depending upon receptor engagement, Opa-mediated interactions can either activate the innate immune system via neutrophil activation or suppress the adaptive immune system through suppression of CD4+ T cells (132). Expression of Opa proteins is phase variable, and Opa+ variants of Gc are selected during male urethritis (64). The number of different Opa proteins expressed by a single gonococcus increases during infection, but Opa proteins are not required for urethral infection (64; 71). Evidence from clinical isolates and studies with experimentally infected mice indicate that the capacity to express Opa proteins allows Gc to be more persistent in the female genital tract and that *opa* gene phase variation may aid in hormonally-regulated host factor selection of Gc during infection (30; 69).

The lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria is a hallmark characteristic of these pathogens. Gc have a lipooligosaccharide (LOS) in

which the repeating O-antigen polysaccharide sugar that is characteristic of LPS is replaced with an oligosaccharide (64). The specific structure of the LOS can vary within and between strains due to phase variable expression of the glycosyl transferases that extend the structure from the core oligosaccharide (55; 177). For example, in experimental gonococcal infection in male volunteers, gonococci isolated at the onset of symptomatic disease carried the gangliosyl LOS moiety, while those isolated at the onset of purulent discharge carried a paraglobosyl moiety. This suggests that the paraglobosyl and gangliosyl moieties are important during colonization of the male urethra (64).

Post-translational modifications to Gc LOS also influence pathogenesis. For example, phosphoethanolamine (PEA) modification at the 4' position of the endotoxic lipid A core aids in resistance to complement-mediated killing by normal human serum (13). This modification, which is catalyzed by the PEA transferase LptA, is also implicated in resistance to small cationic antimicrobial peptides (78). Sialylation of the terminal galactose of Gc LOS also increases resistance to normal human serum and can affect the infectivity of a strain (19; 112; 139). Evidence from male volunteers indicates that LOS sialylation decreases infectivity of Gc, possibly due to blocking Gc invasion through the asialoglycoprotein receptor (53). Sialylation of LOS during infection may aid extracellular bacteria in evading defenses encountered over the course of infection, such as blocking opsonic uptake and killing by human neutrophils (64; 142; 176).

1.3a2 Transferrin and Lactoferrin Receptors

Iron sequestration within the human body limits its availability to bacterial pathogens. Gc circumvents this innate defense through use of a two-component receptor encoded by *tbpA* and *tbpB* to bind human transferrin and acquire sequestered iron for

growth. Uptake of human lactoferrin occurs similarly through a receptor encoded by *lbpA* and *lbpB*. However, some strains of Gc carry a deletion in *lbpA,* which abrogates expression of the lactoferrin binding protein A (6). Experimental evidence demonstrates that either a functional transferrin or lactoferrin receptor is required for human infection in male volunteers. Furthermore, a strain that expresses both receptors has a biological advantage over expression of the transferrin receptor alone. (33; 64).

1.3a3 IgA1 Protease

Gc expresses IgA1 protease, which cleaves human IgA1 at a dense, proline-rich consensus sequence within the hinge region (61). IgA1 protease is also capable of cleaving human lysosome/late endosome-associated membrane protein 1 (h-lamp-1) at a similar proline-rich sequence (61). This bacterial defense promotes intracellular survival in human epithelial cells *in vitro* (92). Additionally, *in vitro* studies have demonstrated that Gc can interfere with programmed cell death, or apoptosis of human monocytic cells, through cleavage by IgA1 protease of tumor necrosis factor-α (TNF-α). This serves as a way of subverting the host innate immune response (15). Despite the indications that Gc IgA1 protease plays a role in infection, IgA1 protease is not necessary for infection of naïve male volunteers. This finding may indicate that this protease is necessary at later stages of infection that cannot be measured in the current model of infection or that it protects against reinfection (64).

1.3a4 LOS/Peptidoglycan-mediated Damage to Fallopian Tube Tissue

Gonococcal adherence to non-ciliated cells of the fallopian tubes causes subsequent shedding of the ciliated cells in fallopian tube mucosa organ culture (100). This system of culture has been used to study the molecular pathogenesis of acute

gonococcal salpingitis and post-salpingitis sequelae. The non-ciliated cells of the fallopian tube epithelium transport the gonococcal cells across the epithelial barrier; however, the ciliated cells are consequently shed. These ciliated cells are necessary to move the ovum down the fallopian tube during ovulation. Loss of ciliation can either prevent the ovum from moving, which leads to infertility, or allow the ovum to be fertilized within the fallopian tube, resulting in ectopic pregnancy. In humans, acute salpingitis can lead to complete loss of ciliary action within the fallopian tubes (43). This loss is attributed to LOS, peptidoglycan, or a combination of the two, which cause increased production of TNF-α, leading to a damaging local response (99; 100).

While all Gram-negative bacteria have developed mechanisms to recycle peptidoglycan (PGL) during cell growth, Gc also releases immunologically significant quantities of 1,6 anhydro monomers that contribute to pathogenesis through killing of ciliated epithelial cells in the fallopian tubes (49). In addition to PGL, gonococcal LOS in organ culture experiments has been shown to localize to ciliated cells and cause complete sloughing by 24 hours post-inoculation (32). This induction of sloughing may provide Gc with access to the sub-epithelial cell compartment beneath the mucosa (43).

1.3b Phase and Antigenic Variation

The ability of *Neisseria* to vary surface antigens through both phase and antigenic variation aids in evasion of the adaptive immune response. Furthermore, variable expression of surface molecules allows Gc to produce functionally different variants. Phase variation is the on/off regulation of gene expression, which results in bacteria that do or do not produce particular surface molecules. This reversible genetic switch occurs at a faster rate than the frequency of spontaneous mutation within a population. This

variation is heritable between generations, but it continues to be reversible in the daughter cell. Phase variable phenotypes in Gc include PgtA-controlled type IV pilin modification, the PilC pilin tip adhesin, the FetA siderophore receptor, opacity (Opa) proteins, and LOS length and structure because of phase variable expression of the *lgtA, lgtC,* and *lgtD* glycosyltransferase genes. Phase variable expression of these phenotypes occurs through slipped-strand mispairing (SSM) during replication within the relevant structural gene (165).

Unlike phase variation, antigenic variation is the expression of different variants of a particular protein within a clonal population (165). In Gc, type IV pilus antigenic variation occurs via recombination events between the pilin expression locus, *pilE* and one of 19 silent *pilS* loci (23). Gc pilin antigenic variation occurs at a rate of 4 x 10^{-3} events per cell per generation (36) and is thought to facilitate evasion of pilus-specific immune responses that could potentially block colonization (137).

1.4 ANTIMICROBIAL RESISTANCE

The discovery of penicillin by Sir Alexander Fleming in 1928 ushered in the modern era of antibiotics. Although Sir Fleming himself was never able to develop the antibacterial mold he had discovered into a mass-produced antibiotic for human use, efforts by scientists in both the United States and England during World War II brought about the advent of the antibiotic era (121). The driving force at the time was the desperate need to ensure an effective and life-saving means of treating battlefield wounds before the D-Day invasion. The World War II effort stimulated drug discovery and development and ushered in the modern antibiotic industry (121). Today, we face a similar threat to our ability to treat infectious disease. While the quote "It is time to close

the book on infectious diseases, and declare the war against pestilence won," once attributed to U.S. Surgeon General Dr. William H. Stewart has been debunked as an urban legend, the sentiment that allowed it to become a well-known (if incorrect) quote in the 20th century has run its course (147). We no longer live in an era where the end of bacterial diseases seems a certainty.

Bacterial adaptation to antibiotics has been extremely successful. Indeed, we exist on a precipice, where the real threat of a future of untreatable bacterial infections looms large. New resistance mechanisms emerge and spread in our increasingly connected global society. There is a growing worldwide threat of antimicrobial resistance (AMR) that includes antimicrobial-resistant fungal, parasitic, viral, and bacterial infections. The emergence of resistant organisms and the antifungal, antiparasitic, antiviral, and antibacterial medications that they render obsolete, threatens to undermine effective modern health care. AMR leads to increased morbidity, prolonged hospitalizations, and in some cases, increased mortality (175). Although numbers are not available worldwide, it is estimated that AMR costs the U.S. health care system between $21 and $34 billion dollars annually (22; 175). The following sections provide a brief overview of the state of AMR in fungi, parasites, and viruses, before focusing on AMR in bacteria.

1.4a Antifungal Drug Resistance

Fungal pathogens are either multi-cellular filamentous molds or single-cellular microscopic yeasts. Antifungal resistance typically refers to invasive *Candida* infections, although there are isolated reports of multi-azole resistant *Aspergillus fumigatus* in Europe (175). Candidiasis can range from superficial infections such as oral thrush to systemic blood infections, which are classified as invasive candidiasis. Over 20 species of

Candida can cause candidiasis. Candidaemia, or bloodstream Candidiasis, is a common cause of vascular catheter-related illness. These infections cost the U.S. an estimated $8 billion yearly, although those numbers do not differentiate the burden of drug-resistant *Candida* (175). The situation, although not as dire as some other forms of AMR, warrants close monitoring. There are three classes of antifungals indicated for candidiasis*:* the azoles, the echinocandins, and the rather toxic polyenes, which include amphotericin B. Resistance to all three of these antifungals has been reported (175). Within *Candida* species, the most common mechanism of azole resistance comes from induction of the *mdr* or *cdr* genes that control the expression of multi-drug resistant efflux pumps. In addition to activation of efflux pumps, the next most common mechanism of antifungal resistance is point mutations in genes encoding the drug target of both azoles and echinocandins, which render the drugs ineffective (120).

1.4b Antiparasitic Drug Resistance

Antiparasitic drugs cover a range of species, from protozoans to arthropods to helminths, and resistance hinders attempts to bring parasitic diseases under control. The protozoa *Giardia duodenalis*, which causes diarrheal disease, and *Trichomonas vaginalis,* which causes the most common non-viral, non-bacterial sexually transmitted infection worldwide, are both treated with nitroimidazoles. Additionally, both are commonly associated with treatment failures resulting from drug resistance (133). The enzymes pyruvate: ferredoxin oxidoreductase and ferredoxin are necessary to activate metronidazole, and are severely downregulated in resistant *T. vaginalis* through changes in the upstream regulatory regions of the genes that encode these enzymes (133; 164). In *G. duodenalis,* downregulation of ferredoxin oxidoreductase expression via gene

rearrangement and electron transport changes as well as by increased efflux of the drug can lead to resistance (133; 164).

Over 40% of the world's population is at risk of malaria, a life-threatening disease caused by the protozoan *Plasmodium*, which is spread by the arthropod mosquito vector *Anopheles* (133; 175)*.* In the absence of a vaccine, treatment with antiparasitic drugs is the cornerstone of the effort to curb malarial disease. Most of the morbidity and mortality in malaria is associated with disease caused by *P. falciparum. P. falciparum* develops resistance to antimalarials through spontaneous mutational events in a single parasite, which are selected during treatment. This single event gives rise to a resistant population that is then able to spread unchecked. This resistance can be the result of multiple mutations or a single *de novo* event (175). The first resistance that arose was against chloroquine, which occurred in the late 1960s in both Southeast Asia and South America and subsequently spread worldwide (133; 175). Pyrimethamine and sulfadoxine were then used to treat resistant strains, but resistance again arose (133; 175). The introduction of mefloquine followed in the 1980s (133; 175); however, resistance emerged in Southeast Asia, this time in Cambodia and Thailand, and spread worldwide (133; 175). Today, malaria is treated with artemisinin combination therapy (ACT), where artemisinin is combined with partner drugs such as amodiquine and pyrimethamine/sulfadoxine. While artemisinin resistance is still not widespread, there have been pockets of resistance identified in Cambodia, Myanmar, Thailand, and Vietnam (175).

Resistance to chloroquine occurs when two mutations, one in the pf*mdr1* gene that encodes an efflux pump, and one in the pf*crt* gene (*c*hloroquine *r*esistance *t*rait), are selected in near concurrence (133). Because two simultaneous mutations are required,

chloroquine resistance arises rarely. However, where it does occur, the resistant strains

spread easily in regions with little to no vector control. Resistance to

pyrimethamine/sulfadoxine similarly requires mutations in two genes: one in the gene

encoding dihydrofolate reductase, and the other in the gene that encodes dihydropteroate

synthase (133). Resistance to mefloquine occurs through mutations that result in multiple

copies of resistance alleles of pf*mdr1*. Field studies have demonstrated that the same

mutations that result in resistance to mefloquine are associated with resistance to

chloroquine (42; 133). Drug resistance in *Plasmodia* correlates with reduced fitness *in

vitro* and in animal models when resistant strains are competed against sensitive wild-

type strains without drug pressure. This interplay is not straightforward; certain resistance

polymorphisms that decrease fitness of *Plasmodium* in the human have been associated

with increased fitness in the mosquito vector. In addition, compensatory mutations that

restore fitness without compromising resistance are suspected in the case of pf*crt,* in

which resistance is associated with the K76T single nucleotide polymorphism (SNP).

Multiple other SNPs always accompany this mutation and are likely to encode

compensatory mutations (130).

Hookworm treatment failures have been reported for *Necator americanus*

infections treated with mebendazole (51). SNPs identified in the gene encoding for the β-

tubulin isotype 1 account for mebendazole resistance (68). Additionally, increased

expression of ATP-binding cassette (ABC) transporter superfamily proteins is associated

with resistance in multiple helminths, including *Schistosoma mansonii, Fasciola

hepatica, Onchocerca volvulus,* and *Haemonchus contortus,* presumably through

increased efflux of drugs from the target cell (68). Veterinary helminthic parasites have

long been treated using a rotating strategy of antihelminthic classes of drugs. With this approach, a particular class of drugs is used for a year and then rotated, based on the theory that if resistance had developed, it would revert when the drug was removed; the less fit resistant strain would be unable to compete (86).

1.4c Antiviral Drug Resistance

While a seasonal vaccine is the recommended prophylactic for human influenza, two classes of drugs are also available for treatment of infected individuals: adamantanes and neuraminidase inhibitors (175). Reports of the first *in vitro* resistance to the adamantanes emerged in the 1970s, and clinical resistance followed (123). Interestingly, the same genetic flexibility that allows the virus to express different combinations of the hemagglutinin (17 subtypes) and neuraminidase (10 subtypes) surface proteins is responsible for antiviral resistance. Widespread resistance to the adamantanes has been reported (175); in contrast, resistance to oseltamivir, a neuraminidase inhibitor, is low but emerged widely against the 2007-2008 seasonal influenza A (H1N1) virus. This outbreak supports the potential for widespread neuraminidase resistance (175).

Members of the herpesviridae family that exhibit antiviral resistance include herpes simplex virus types 1 and 2 (HSV), varicella-zoster virus (VZV), and cytomegalovirus (CMV) (52). Acyclovir and ganciclovir inhibit the viral DNA polymerase of HSV, VZV, and of CMV, respectively by using the phosphorylation activity of the viral thymidine kinase (TK) to phosphorylate the drugs to their active triphosphate form. The active phosphorylated drugs then act as competitive inhibitors of viral DNA polymerase.

HSV drug resistance occurs through either mutations in the gene encoding the viral TK that change the substrate specificity of the viral TK, or mutations in the gene encoding the viral DNA polymerase that change the substrate specificity of the polymerase. Some examples of these mutations include additions or deletions in extensive runs of G's and C's within the TK gene that cause frameshift mutations, as well as SNPs in the catalytic site of the enzyme. In VZV, where the runs of G's and C's are fewer, premature stop codons contribute to resistance. Mutations in the viral DNA polymerase that confer resistance while maintaining function occur in both HSV and VZV. These mutations are often clustered within certain conserved regions of the gene. CMV resistance to ganciclovir occurs in 15-38% of patients treated for greater than three months. Resistance is due to either reduced accumulation of the triphosphate form of the drug in virus-infected cells (the most common form of resistance) or mutations in the viral DNA polymerase gene. Preliminary studies in cell culture have demonstrated that HSV and CMV carrying resistance mutations in the viral DNA polymerase replicate less frequently than wild-type virus; this finding demonstrates an *in vitro* fitness disadvantage accompanies these resistance mechanisms. This hypothesis has not yet been tested in an *in vivo* model (52).

Human immunodeficiency virus, or HIV, systematically attacks cells of the human immune system until function weakens to the point that the patient develops clinical acquired immunodeficiency syndrome (AIDS). WHO and UNAIDS estimated that in 2011, there were 2.5 million new infections world-wide to bring the year-end total of people living with HIV infection to 34 million. Mortality associated with the disease accounted for 1.7 million deaths, 230,000 of which were children (175). The burden of

AIDS morbidity and mortality is felt worldwide, although it is particularly pronounced in areas of economic distress, where access to antiretroviral therapy (ART) is limited. ART effectively treats infection and prolongs the lifespan and quality of life of infected patients. Unfortunately, in the United States alone, estimates predict that 50% of those treated with ART are infected with a viral strain that is resistant to at least one antiretroviral drug (29; 124). ART works to slow viral replication, thereby decreasing viral load and giving the compromised immune system a chance to mount a defense (175). Resistance occurs frequently, mainly because of the rapid and error-prone replication of the virus.

ART against HIV consists of the use of drugs belonging to one of four classes: nucleotide/nucleoside analogues, non-nucleoside reverse-transcriptase (RT) inhibitors, protease inhibitors, and fusion inhibitors (29). Nucleotide and nucleoside analogues act as viral replication terminators of the nascent viral DNA through incorporation and premature termination. Resistance can occur through mutations that either promote excision of the terminator or block incorporation of the analogue. RT inhibitors bind HIV-1 RT to block polymerization of viral DNA. Mutations, often SNPs, can confer resistance by blocking the binding of the drug to the viral RT. Protease inhibitors bind the active site of HIV-1 protease to block activity, and like RT inhibitors, resistance mutations can block the binding of the drug to the protease. Fusion inhibitors block viral fusion to host cells by inhibiting the glycoprotein 41 (gp41)-mediated fusion of virus to cell. Resistance mutations in the HR1 domain of gp41 stimulate fusion and block the drug (29).

HIV accrues multiple mechanisms of resistance over time, which can lead to failure of more than one class of drugs during ART. However, this accumulated resistance is also associated with a fitness cost (29). Indeed, viral replication becomes compromised because of mutations in vital viral proteins. Some of these resistance mutations are at least partially compensated for by compensatory mutation(s). Researchers discovered compensatory mutations in a patient treated solely with a protease inhibitor. Over the course of therapy, mutations arose that increased resistance to protease inhibitors but decreased viral fitness. However, as treatment continued, subsequent secondary mutations restored fitness while maintaining resistance (113).

1.4d Antibacterial Drug Resistance

Bacteria are resistant to antibiotics by two mechanisms: intrinsic resistance to the particular antibiotic and acquired resistance through chromosomal mutation or horizontal gene transfer. Intrinsic resistance is a result of a particular structural or functional characteristic of the bacterium that blocks the antibiotic. In simplest terms, if a particular bacterial species lacks the target of the antibiotic, it is intrinsically resistant. For example, daptomycin is an antibiotic that is active against the cytoplasmic membrane of Gram-positive, but not Gram-negative bacteria. This is due to differences in the anionic phospholipid content of Gram-negative bacteria that hinder the ability of the drug to insert into the membrane in a Ca^{2+}-dependent manner (18). Acquired resistance, on the other hand, is developed resistance to a particular antibiotic or class of antibiotics by modification of the target, modification of the antibiotic or decreased access to the target.

1.4d1 Modification of the Target

Modification of a drug target can occur through either mutation of the gene encoding the target or protection of the target protein (18). Examples of mutations of the target gene include point mutations in *gyrA*, which encodes the A subunit of DNA gyrase. These mutations can increase resistance to the fluoroquinolones by altering the region of GyrA that binds this class of antibiotics. A second example are mutations that encode amino acid substitutions in PBP2, or PenA that confer resistance to the ESCs by altering drug binding capability (18; 84; 155).

Examples of mechanisms that protect the target protein include methylation of 16S rRNA and 23S rRNA, which prevent multiple drugs that inhibit protein synthesis from binding to these targets. The *cfr*-encoded chloramphenicol-florfenicol resistance methyltransferase, which confers resistance to multiple drugs in both Gram-positive and Gram-negative organisms, is one example of these resistance-promoting enzymes (18). This gene is carried on a plasmid, which serves to facilitate the spread of resistance. Similarly, the *armA* gene of the Enterobacteriaceae encodes a methyltransferase that methylates ribosomes to confer aminoglycoside resistance. Other examples include: genes that encode proteins that protect topoisomerase IV and DNA gyrase from the action of the quinolones; alterations in bacterial LPS that confers protection to antimicrobial peptides like polymyxin B; and mutations that change the charge of the phospholipids that make up the cytoplasmic membrane, thereby preventing binding of daptomycin, which targets anionic phospholipids (18).

1.4d2 Modification of the Antibiotic

β-lactamases cleave the β-lactam ring of β-lactams such as penicillin through hydrolysis, leading to drug inactivation. The first β-lactamase, penicillinase, was

discovered in the 1940s. Since then, many others have emerged. Second and third generation penicillins were developed to resist β-lactamases, and the extended-spectrum β-lactamases followed. Today, the extended-spectrum cephalosporins are used because they are refractive to most β-lactamases. Since the discovery of the first β-lactamase, many other enzymes that inactivate drug targets through hydrolysis have been discovered. These enzymes target a wide range of antibiotic classes, including penicillin and the other β-lactams, aminoglycosides, macrolides, and phenicols (18).

Additionally, bacteria can modify the antibiotic through addition of a chemical group that then provides steric hindrance at the active site of the antibiotic. Acetyltransferases, phosphotransferases, and nucleotidyltransferases confer resistance to aminoglycosides, a class of large antibiotics that contain exposed regions that are ripe for chemical modification (18).

1.4d3 Decreased Access to the Target

Decreased access to the antibiotic target can occur through either reduced permeability during influx of the drug, increased efflux, or a combination of both. Part of this decreased access can be explained in Gram-negative bacteria through intrinsic resistance conferred by an outer membrane not present in Gram-positive bacteria. Drugs cross the outer membrane through non-specific porin channels, and in *Klebsiella pneumoniae,* expressed porin variants have been linked to outbreaks of antibiotic resistant infections (18). In Gc, mutations in *porB*$_{1b}$, which encodes one of the two subtypes of the major Gc porin, lead to decreased antibiotic uptake, and contribute to resistance to multiple classes of antibiotics (87).

Bacterial efflux pumps actively transport antibiotics out of the cell. While the Tet efflux pumps are specific to tetracycline, the multidrug resistance (MDR) efflux pumps can efflux several different drugs, including ones that are structurally dissimilar. In both cases, resistance occurs through overexpression of the efflux pump. Though most are chromosomally encoded, some MDR efflux genes have been mobilized onto plasmids, which contribute to the spread of resistance. The well-studied MDR pumps, the resistance nodulation division (RND) pumps, are tripartite systems that are controlled by expression of a repressor protein. Resistance occurs through de-repression of the pump-encoding operon, often through mutations that abrogate expression of the repressor. Increased expression may also occur in response to stimuli in the host environment. For example, in Gc, expression of the multiple transferable resistance pump *mtrCDE* is increased under conditions of iron limitation *in vitro,* a condition that is typically encountered during host infection (18).

1.5 ANTIBIOTIC RESISTANCE IN *NEISSERIA GONORRHOEAE*

Antibiotic treatment of infected individuals and their sexual contacts is a critical and primary control measure for gonorrhea; however, this strategy is threatened by the rapid evolution of resistance in Gc (Figure 1). Gc susceptibility to ceftriaxone, the last remaining option for antibiotic monotherapy for gonorrhea, has decreased globally over the last decade (108). Without effective and innovative new drugs to intervene, the emergence of ceftriaxone-resistant (Cro^R) strains will severely hinder our ability to treat infection (162; 163). Indeed, the first strain with high-level ESC resistance was isolated and characterized in Kyoto, Japan, in 2009. Furthermore, transmission of a high-level ceftriaxone-resistant strain between two male sexual partners in Spain was recently

described (24; 116). Dual antibiotic therapy is now recommended in the USA and Europe (27). Gc is a remarkably agile microbe that has co-evolved with its human host. Thus, it should be no surprise that, along with an impressive repertoire of mechanisms for evading host defenses, this pathogen has also developed multiple modes of evading the wide range of antibiotics historically employed against it.

Furthermore, Gc is a naturally competent bacterium. As previously discussed, Gc only takes up DNA that carries the 10-base pair DUS, which is strewn throughout the genome of Gc and other *Neisseria* species (2). The expression of Type IV pili is necessary for uptake of DNA in Gc (2; 28). The recently identified DUS receptor, ComP, triggers DUS-specific transport across the outer membrane and into the periplasm (17). In the current working model of DNA competence in Gc, prepilin peptidase (PilD) processing of the pilin subunits PilE and ComP forms the pseudopilus, which uses the Type IV secretion machinery to transport the incoming DNA across the outer membrane through the secretin PilQ (83). The secretin acts as a channel to bring homotypic dsDNA or ssDNA into the cell via the disassembly of the pilin subunits and subsequent retraction of the pseudopilus. ComE binds DNA in the periplasm and delivers it to ComA, a channel that allows it to cross the cytosolic membrane into the cytosol. During this transfer, one strand of the double-stranded DNA is digested and released into the periplasm (28).

The ability to take up exogenous DNA only speeds the rapidity of the transfer of antibiotic resistance. Until we are able to develop an effective vaccine, treatment is driven by the constant push-pull of human antibiotic use and bacterial acquisition of antibiotic resistance determinants.

The sections that follow contain a brief description of the mechanisms by which Gc resists antibiotics that were previously recommended for routine treatment of gonorrhea.

Figure 1. Timeline of development of antibiotic resistance in *N. gonorrhoeae.*

        Mechanisms of antibiotic resistance have evolved and spread for each class of antibiotic introduced against gonorrhea. The first ceftriaxone-resistant strains of Gc were isolated in 2009 and 2010. B-lactam antibiotics are labeled in red. All other antibiotics are labeled in black. This figure is modified from (163).

Antibiotic Introduced

Sulfonamides   Spectinomycin   Fluoroquinolone
Penicillin   Tetracycline   Azithromycin
Streptomycin   **Cephalosporins (Ceftriaxone (CRO)/Cefixime)**
Erythromycin

1936 1940's 1950's 1960's 1970's 1980's 1990's   2007 **2009 (CRO^R H041)**
                                                                  2010 (CRO^R F89)
                                                                  2012
                                        Erythromycin   Fluoroquinolone
            Sulfonamides   Azithromycin

Antibiotic Removed   **Penicillin**   **Cefixime**
                              Tetracycline
                              Spectinomycin

24
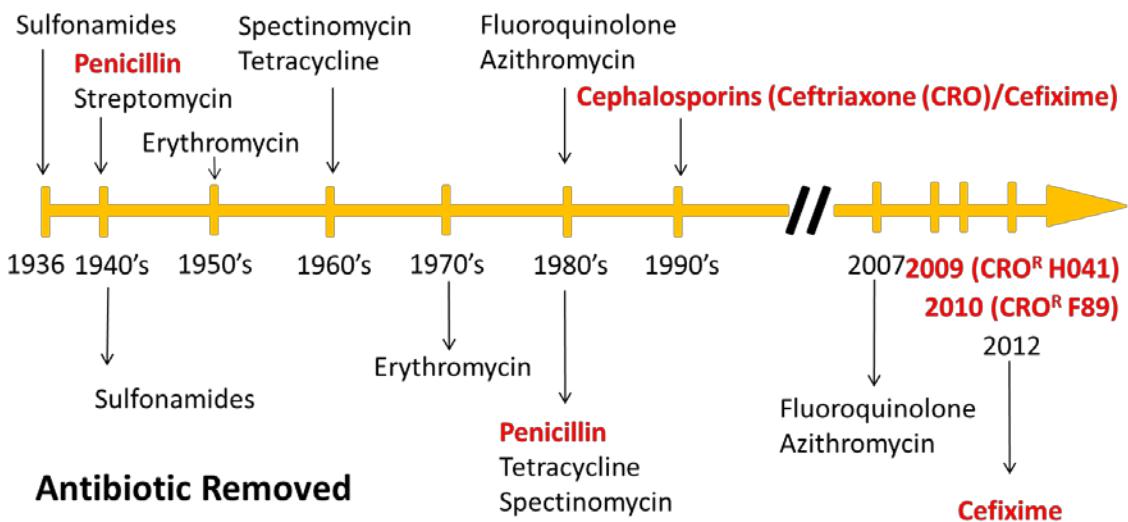
1.5a Sulphonamides

Early remedies for Gc infection were ineffective at best. In the 1890's a silver-based compound, protargol, was used as chemotherapeutic (163). This compound was not particularly effective, and was followed by urethral irrigation as the recommended treatment (87). It was the advent of sulphonamides, or sulpha drugs in the 1930s that began to turn the tide in the fight against Gc. Sulphanilamide was discovered in 1935, and cured 80-90% of gonorrhea cases. However, by the mid-1940's, almost all circulating strains were clinically resistant (87; 163). For a time in the 1970s, combination therapy of sulfamethoxazole and trimethoprim was used to treat Gc infection, but the regimen required multiple doses to be effective (87; 169).

Sulfa drugs inhibit DNA precursor synthesis by blocking the enzyme dihydropteroate synthase, an enzyme unique to bacteria that is involved in folate biosynthesis. Trimethoprim also targets this pathway to block dihydrofolate reductase, an enzyme that is necessary to synthesize pyrimidine thymidylate (169). When trimethoprim is used with sulfamethoxazole, the combination therapy provides synergistic obstruction of two steps within the folate synthesis pathway of Gram-negative organisms.

Sulfa drugs work as competitive inhibitors of *p*-aminobenzoic acid within the active site of dihydropteroate synthase (76). Resistance can occur when Gc overproduces *p*-aminobenzoic acid, which dilutes out the ability of the sulfa drug to block binding at the active site. In addition, mutations to the gene that encodes dihydropteroate synthase can reduce affinity of the enzyme for the sulfa drug (76; 87). Resistance to trimethoprim appears to be more intrinsic; trimethoprim does not have a strong affinity for Gc dihydrofolate (76). As early as the 1940s, it was evident that another drug was needed to replace sulfa drugs in effective treatment of Gc infection.

25

1.5b Penicillin

Penicillin was introduced in 1943 and was touted as the miracle cure for gonorrhea. One dose had the ability to clear infection. In a story that would become sadly familiar for gonorrhea, acquisition of plasmid-mediated mechanisms of resistance and chromosomal mutations led to the end of penicillin effectiveness by the 1980s (87).

Penicillin is the first β-lactam antibiotic discovered, so named for the β-lactam ring. The β-lactam ring is a cyclic amide with the nitrogen atom attached to one of four carbon atoms and is bonded to a five-membered ring that contains a sulfur element (169). These antibiotics work on cell wall biosynthesis by blocking the transpeptidation reaction. During cell wall synthesis, the peptide strands of the nascent peptidoglycan are enzymatically cross-linked by transpeptidases to provide mechanical strength to the mesh-like network of peptidoglycan. This cross-linking allows the bacterium to withstand osmotic pressure that would otherwise destroy it (150; 169). The transpeptidases are also known as penicillin-binding proteins (PBPs). PBPs have both transpeptidase and carboxypeptidase activity during the catalysis of the terminal steps of peptidoglycan synthesis. PBPs are also active-site serine hydrolases, meaning that the serine at the active site acts as a nucleophile, and is first to cleave the amide bond between the terminal D-alanine in the oligopeptide chain (carboxypeptidation) (150; 169). During this release, an acyl-O-transpeptidase intermediate is formed (169). In the second reaction, acyl transfer to the DAP_3 or $Lys_3 C_6$ amine group, a nucleophile catalyzes the release of the free enzyme and generates the isopeptide bond that forms the cross-link (169). This transpeptidation does not create any new peptide bonds; rather it cleaves one during the carboxypeptidation and makes another bond during the cross-linking of Lys-D-Ala or DAP-D-Ala (169).

26

B-lactam antibiotics acylate PBPs to block these enzymes by forming an acyl-enzyme stable intermediate. This block in cell wall synthesis is bactericidal. Although Gc has four PBPs, only PBP1 and PBP2 are considered critical to cell wall synthesis. PBP1 catalyzes transpeptidation during cell elongation while PBP2 catalyzes transpeptidation during cell division (150). Penicillin acylates PBP2 at an approximately 10-fold greater rate than PBP1. This is considered the step where the antibiotic blocks cell wall synthesis (129).

Acquisition of plasmids that carry TEM-1-type β-lactamase, capable of hydrolysis of the β-lactam ring, was the first mechanism of penicillin resistance in Gc. The plasmids were easily mobilized, and several different types spread worldwide (87). Plasmid-borne resistance was followed by the evolution of chromosomally-mediated penicillin resistance, which was acquired in a temporal step-wise fashion.

Acquisition of chromosomal mutations that confer penicillin resistance is a systematic procedure, with each individual resistance determinant adding to the overall resistance of the bacterium. First, a mutation in the *penA* gene, which encodes PBP2, is acquired. This mutation is typically an insertion after residue 345 of an aspartate near the active site of the enzyme (20). Acquisition of mutations in either the *mtrR* repressor gene, which controls expression of the *mtrCDE* operon, or its promotor region, follows. Substrates of the MtrC-MtrD-MtrE active efflux pump include antimicrobial peptides, hormones, bile salts, fatty acids, and multiple classes of antibiotics. Several mutations can lead to de-repression of the pump, but most commonly is a simple single base pair deletion in the promotor region of *mtrR* (50; 166). The third step is acquisition of a mutation in the *penB* gene, which encodes the major Gc porin, called PorB$_{1b}$. Gc strains

express either the *porB₁ₐ* or the *porB₁ᵦ* allele to produce PorB$_{1a}$ or PorB$_{1b}$ porins, which differ antigenically. Only the PorB$_{1b}$ serotype is involved in penicillin resistance. The *penB* mutation(s) result in one or two amino acid changes, which in the presence of the *mtrR* mutation, lead to increased penicillin resistance (118). It is unknown what the exact interaction is between the *penB* mutation and the *mtrR* mutation, but both mutations must be present for the *penB* mutation to have an effect (138). The final step necessary for high-level penicillin resistance is a substitution of Leu$_{420}$ for proline in the transpeptidase domain of the *ponA* gene, which encodes PBP1 (129).

By 1989, penicillin was no longer recommended for treatment of gonorrhea (163). It is important to note that while penicillin was effectively removed from treatment almost 30 years ago, the chromosomal mutations that led to penicillin resistance have remained in circulation (163).

1.5c Tetracycline

Introduction of penicillin to treat gonorrhea was quickly followed by tetracycline, which was initially used to treat patients with a penicillin allergy. Tetracycline prevents bacterial protein synthesis in a bacteriostatic manner. This is accomplished by blocking the binding of the aminoacyl-tRNA to the acceptor (A) site of the 30s ribosome, thereby ending protein synthesis without peptide bond formation (169). Tetracycline resistance occurs through modification of the target, whereby a V57M point mutation in the *rpsJ* gene, which encodes ribosomal protein S10, allows the protein to block access of tetracycline to the 30s ribosome binding site. Furthermore, mutations already described in *penB* and the *mtr* locus may confer decreased susceptibility to tetracycline through decreased access of tetracycline to the cell and increased active efflux, respectively (65).

Plasmid-mediated tetracycline resistance also occurs. Plasmids carrying the *Streptococcal*

tetM gene encode a protein that causes tetracycline release by the ribosome, and thereby

reduced susceptibility (87). High-level resistance to tetracycline was reported in 1985,

and the drug was removed from recommended treatment in 1986 (163).

1.5d Spectinomycin

Spectinomycin, like tetracycline, acts on the 30s ribosomal subunit to block

protein synthesis. When it binds to the 30s ribosomal subunit, it blocks the 16s rRNA-

mediated transfer of the aminoacyl-tRNA from the A site to the P site during protein

peptide elongation (159). In Gc, resistance to spectinomycin occurs through a SNP in the

16s rRNA. A novel mutation in the ribosomal protein S5 can also confer spectinomycin

resistance as occurred in a case of treatment failure in Norway in 2010 (159).

Spectinomycin was introduced for treatment in the 1960s, but was rapidly removed as a

recommended treatment because of studies that demonstrated that during widespread use,

high-level resistance spreads rapidly (87).

1.5e Aminoglycosides

Like tetracycline, the aminoglycosides came into use as an alternative to

penicillin. The first aminoglycoside, streptomycin, was discovered in 1944 (169). These

antibiotics target the 16s rRNA and protein synthesis, mainly at the A site of the 30s

ribosomal subunit, where aminoacyl-tRNA binds (169). Further work has shown that a

mutation in *rpsL*, which encodes ribosomal protein S12, accounts for streptomycin

resistance in some strains of Gc (149). Resistance does arise rapidly to this class of

antibiotics and aminoglycosides are not considered first-line treatment (163). Of note,

however, recent work has shown that a single-dose of gentamicin cleared Gc infection in

91.5 % of treated subjects (41). While the results did not meet CDC standards for disease

clearance, further work demonstrated that combination therapy of gentamicin with

azithromycin cleared disease in 100% of study participants (81). This important study

supports dual therapy with gentamicin and azithromycin as a potential alternative for

patients who are unable to tolerate cephalosporin treatment or are colonized with

ceftriaxone-resistant Gc. The propensity of Gc to develop resistance to aminoglycosides,

however, means this alternative therapy is not recommended to supersede the current

regimen.

1.5f Macrolides

Erythromycin, the original macrolide antibiotic, was introduced in the 1950s to

treat gonorrhea. However, by the 1970s it was removed from the treatment regimen

because of widespread resistance (163). Erythromycin is produced by the streptomycete

Saccaropolyspora erythraea. Development of azithromycin followed, which is a

derivative extended-spectrum macrolide. Azithromycin is a semisynthetic molecule

modified with an extended 15-membered macrolide moiety and an added nitrogen (169).

Macrolide antibiotics have dual action, both of which affect 23S rRNA. Binding of

macrolides to the 23S rRNA blocks the nascent peptide from entering the exit tunnel,

causing early release of the peptidyl-tRNA intermediate. Additionally, macrolide

interactions with the 23S rRNA block assembly of the 50S subunits (169). Macrolides are

a substrate of the *mtrCDE* efflux pump, and resistance occurs due to mutations that cause

de-repression of the pump. Other mechanisms of macrolide resistance include mutations

to the peptidyl transferase loop of domain V of the 23S rRNA and expression of the

genes *ermB, ermC* and *ermF,* which encode methylases that can modify the 23S rRNA

target (35; 111; 126). These particular methylases are carried on conjugative transposons, and spread occurs rapidly (126).

Azithromycin is of particular note, as it is the currently used with ceftriaxone to treat Gc infection. While the recent use of azithromycin with ceftriaxone in dual therapy for Gc infection helps to prevent treatment failures, in settings where azithromycin has been frequently used alone, treatment failures have occurred. Additionally, there has been recent spread of high-level resistance in Scotland, Italy, the United States, Australia, and Sweden as a result of mutations in domain V of the 23s rRNA, which have not been seen previously (157; 162).

1.5g Fluoroquinolones

Fluoroquinolones, specifically, ciprofloxacin and ofloxacin, began to be used to treat gonorrhea in the mid-1980s as an alternative to ceftriaxone (87; 163). Despite reports of the spread of fluoroquinolone resistance in Asia, and detection of the first ciprofloxacin-resistant Gc in the US in Hawaii in 1991, ciprofloxacin became a recommended first-line treatment for gonorrhea in 1993 (163). These antibiotics work by targeting DNA Type II topoisomerases, specifically DNA gyrase, which adds negative supercoils into the DNA and eases positive supercoils, and topoisomerase IV, which removes both positive and negative supercoils from DNA (169). Acquisition of high-level fluoroquinolone resistance occurs in a stepwise fashion. Point mutations in *gyrA*, which encodes the A subunit of DNA gyrase, leads to intermediate levels of resistance. When point mutations in *parC*, which encodes the A subunit of topoisomerase IV, accompany these *gyrA* mutations, high-level resistance occurs (16; 140; 151). Due to the

spread of these resistance determinants, the use of fluoroquinolones as a first-line

treatment for gonorrhea was no longer recommended in 2007.

1.5h Extended-Spectrum Cephalosporins

Cephalosporins were first used to treat Gc infection in the early 1980's:

ceftriaxone in 1980 and cefixime in 1983. They were not the sole recommended first-line

treatment for the disease until 2007, as the efficacy of ciprofloxacin was on the steep

decline (163). Cephalosporins, like penicillin, are β-lactam antibiotics that work on the

terminal steps of cell wall biosynthesis; they bind the PBPs and lock them into an acyl-

stable intermediate that cannot finish the transpeptidation reaction. The first generation

cephalosporins were effective against Gram-positive bacteria. Side chain modifications

created the second-, third- and fourth-generation cephalosporins. These modifications

allow the ESCs to better penetrate the cell through porins, and increase the range of

activity to a broader spectrum, which includes both Gram-positive and Gram-negative

activity in the case of the third- and fourth-generations (169). The greater coverage of

Gram-negative organisms is a result of an increased ability to diffuse through the porins,

greater binding affinity for the PBPs, and most importantly in terms of resistance,

refraction to the activity of β-lactamases (169).

ESC resistance began to spread in the 1990s from Japan. Unfortunately, during

this time ceftriaxone was banned in Japan and as a result, only oral cephalosporins were

used for treatment. Because the dosing was longer for many of the oral ESCs, cefixime,

the most powerful of the oral ESCs, was used at a suboptimal concentration of 300 grams

for single-dose therapy, versus the 1 x 400 gram dose recommended worldwide. These

factors encouraged incrementally increasing minimum inhibitory concentrations (MICs)

to ESCs across Japan. This is despite the fact that ceftriaxone was reintroduced to the treatment regimen in the early 2000s (162). By 2006, cefixime was removed from recommended treatment because of treatment failures in Japan and later Europe (160; 161; 178); ceftriaxone was the sole remaining cephalosporin for monotherapy (162).

In 2009, the first strain of Gc that was refractive to ceftriaxone treatment, strain H041, was isolated from the pharyngeal space of a female commercial sex worker in Kyoto, Japan (117). The MIC of ceftriaxone against this strain was 2 µg/mL, and it was extensively resistant to several other antibiotics. The patient was treated with a standard dose of 1 g of ceftriaxone intravenously, but was still Gc-positive two-weeks later. The patient received an additional dose of ceftriaxone, but was still culture positive two weeks later. At that time, the doctors treated the patient again, but were unable to determine whether she was still infected from the index case, or whether reinfection had occurred. It took three months before the patient was free of infection. Of note, further MIC testing revealed that this extremely drug-resistant strain was actually susceptible to spectinomycin. Fortunately this drug was available to treat her (117). Isolation of strain F89 in Quimper, France in 2010 followed this case. F89 was a urethral isolate taken during test of cure from a man who had sex with men (MSM). Interestingly, although F89 was resistant to ceftriaxone, it was discovered as a treatment failure of cefixime. Gentamicin eventually cleared the disease (158).

Thus far, strains belonging to one of two multi-locus sequence types (MLST) have been responsible for all of the cefixime treatment failures as well as many of the recently emerged ceftriaxone failures. Both MLSTs originated in Japan. H041 falls within MLST 7363, and F89 within MLST 1901. MLST 1901 includes NG-MAST

ST1407, which is the strain that is currently responsible for most of the Gc AMR

circulating in Europe (162).

The recommended treatment for Gc infection in the United States was updated in

2012 to reflect the current state of antimicrobial resistance, and consists of a 250

milligram intramuscular dose of ceftriaxone plus either 1 gram of azithromycin as a

single oral dose or 100 milligrams of doxycycline orally twice daily for 7 days where

azithromycin is contraindicated (27).

High-level ceftriaxone resistance, like many other forms of AMR, is

multifactorial. Like penicillin resistance, mutations within *penA* are necessary for

acquired, high-level ceftriaxone resistance. Decreased susceptibility to ESCs occurs

through the acquisition of Ala-501 mutations in the *penA* allele, or through the

acquisition of a mosaic *penA* allele. Mosaic *penA* alleles are a result of the intraspecies

sharing of genetic material through horizontal gene transfer. Several commensal

Neisseria sp. such as *N. sicca, N. flavescens, N. perflava, N. cinerea,* and *N.*

polysaccharea, inhabit the oropharyngeal space. Uptake and subsequent recombination of

partial *penA* alleles from commensal species at the site of infection can result in an

altered *penA* allele. These alleles can encode more than 70 amino acid substitutions

(162). Within this large background of mutations, epistatic control exists such that in at

least one case, the mutations G545S, I312M, V316T, and to a lesser extent, N512Y result

in high-level resistance only when eleven other mutations within the *penA* allele are also

present. The crystal structure suggests that three of these four mutations may alter the β-

lactam-binding pocket (162).

Mosaic *penA* alleles can be classified by the mutational pattern present. Mosaic *penA* allele pattern types were first described by Ito *et al.* to differentiate between the different mosaic alleles of a set of strains isolated in Japan with reduced susceptibility to oral cephalosporins and other beta-lactam antibiotics. The initial ten PBP2 mosaic patterns were numbered I through X (67). The novel *penA* allele in strain H041 differs from the previously described mosaic pattern X by twelve amino acids (162). Tomberg *et al.* showed that three of those mutations, A311V, V316P, and T483S, are necessary for high-level ceftriaxone resistance in this strain (155). The A311V and V316P mutations, like the I312V (also present in H041) and V316T mutations previously described, alter the β-lactam binding pocket (155; 162). The T483S mutation, unique to strain H041, affects PBP2 acylation by loss of the methyl group present in threonine (155). The mosaic *penA* allele of strain F89 most closely resembles mosaic pattern XXXIV, and differs only by the A501P mutation, which, in the background of the intermediate resistance conferred by the XXXIV mosaic allele, confers high-level resistance (158) (Figure 2).

In addition to a mosaic *penA* allele, ESC resistance also requires *mtrCDE* de-repression as discussed previously, as well as the *penB* mutation. The *ponA* mutation is not involved in ESC resistance (Figure 3). However, these multiple mutations do not fully account for the high-level clinical resistance observed in strains H041 and F89. Furthermore, data agree that there is an as of yet unidentified "factor X" that is also necessary (162).

Figure 2. Critical residues for ceftriaxone resistance within mosaic H041 and F89 *penA* alleles.

Black boxes indicate conserved regions of the enzyme. (A) The residues necessary for high-level ceftriaxone resistance in the *penA41* allele of strain H041 are shown in orange. (B) The single residue necessary for high-level ceftriaxone resistance in the *penA89* allele of strain F89 is shown in orange (116; 154; 155; 158).

A   **H041**

penA41

B

F89
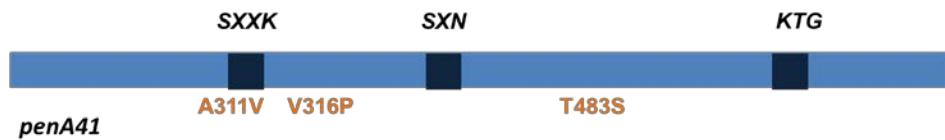
penA89
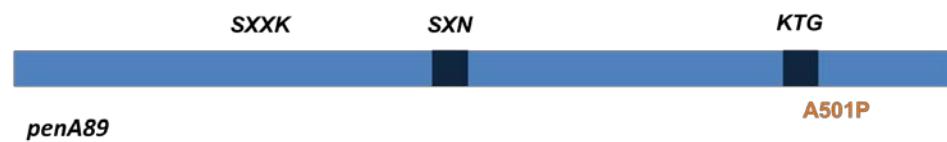
Figure 3. Chromosomally-mediated extended-spectrum cephalosporin resistance.

Chromosomally-mediated ESC resistance is multi-factorial, with several steps leading to high-level resistance. First, amino acid substitutions in PorB$_{1b}$ lead to intermediate levels of ceftriaxone resistance (*penB* mutation). Mutations that de-repress the *mtrCDE* operon, which encodes the MtrC-MtrD-MtrE active efflux pump, act to activate the *penB* mutation and increase drug efflux from the cell. Finally, mosaic *penA* alleles, which encode PBP 2, that result from recombination with different regions of the *penA* gene from other *Neisseria* species, lead to high-level cephalosporin resistance. Figure is modified from (162).

Outer Membrane

Antibiotic Efflux

Ceftriaxone

Overexpression Activates PorB1B Mutations

PorB_{1B}

Decreased Influx

MtrCDE Efflux Pump

Decreased Inactivation

PBP 1

Mosaic PBP 2

Cytoplasmic Membrane
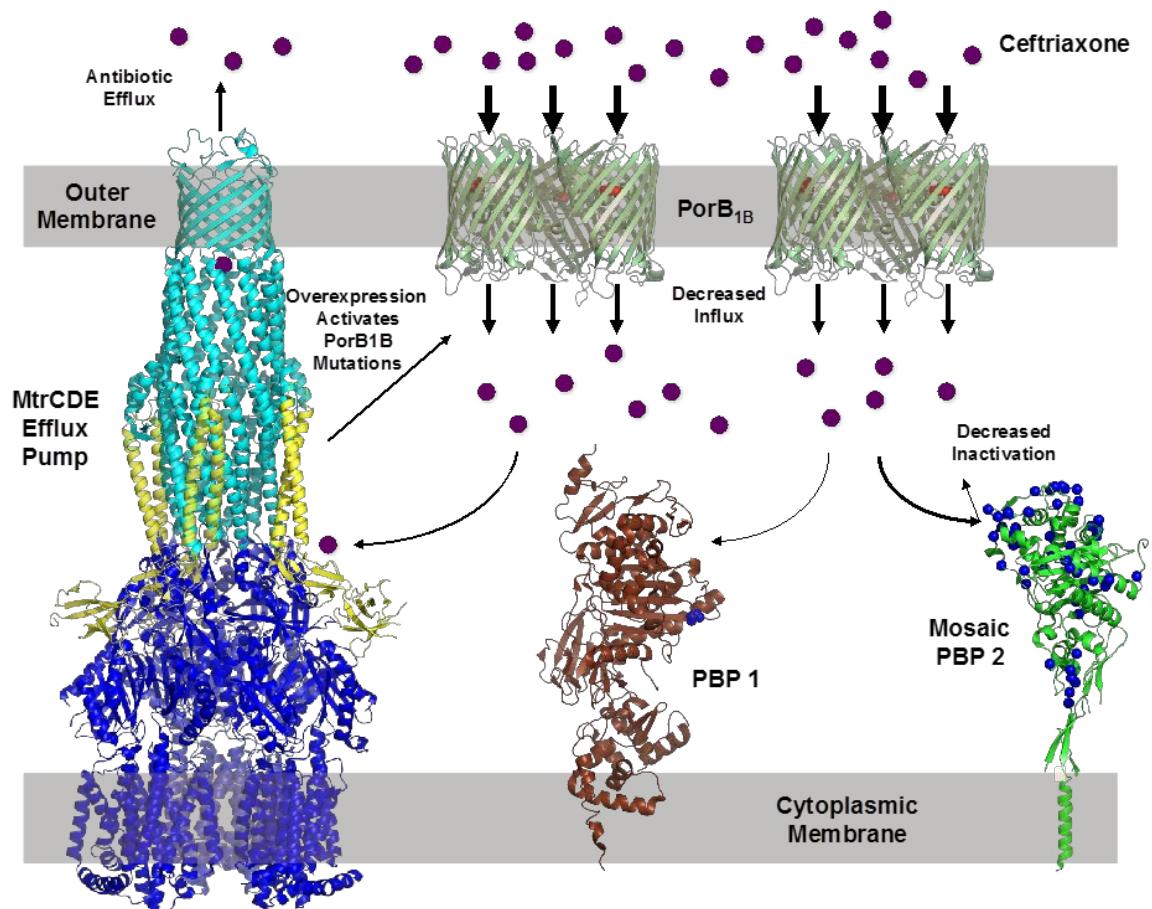
1.6 RESISTANCE AND FITNESS

As discussed earlier, the relationship between antibiotic resistance and microbial fitness can influence the spread of resistance (Figure 4A). Laboratory modeling of the relationship between resistance and fitness is based on comparison studies between two isogenic strains that differ only in the particular gene that confers resistance (Figure 4B). These studies most often show that a fitness cost accompanies antibiotic resistance because the genetic determinants that confer resistance can alter critical functions of the bacterial cell, such as cell wall formation, ribosomal function, and metabolic processing (9). Resistance determinants may also have a neutral or positive effect on fitness.

An example of resistance mutations that reduce microbial fitness are *gyrA* mutations in fluoroquinolone-resistant strains of *Salmonella enterica* serotype enteritidis, which result in altered colony and bacterial morphology, decreased growth rates, less motility, and reduced invasiveness in comparison to wild-type bacteria (115). Other mutations, such as the *rpsL* mutation that confers streptomycin resistance in Gc, confer no biological cost or benefit (this study, (9)). These mutations are biologically neutral. In contrast, *Staphylococcus aureus* strains with mutations in the *mgrA* transcriptional regulator lead to increased expression of the NorB active efflux pump, which confers resistance to multiple antibiotics. Strains carrying this mutation have a marked fitness benefit in a murine abscess model of infection (39).

Compensatory mutation is one mechanism to ameliorate the biological stress conferred by antibiotic resistance, and resistant bacteria with compensatory mutations may be more genetically stable in the environment. Compensatory mutations occur more frequently than reversion mutations, due to the fact that there is a larger mutational target (i.e. the entire genome) for compensation, as opposed to just the site of the original

mutation necessary for true reversion (9). Interestingly, after establishment of resistance in a population, there is scant clinical evidence to show that decreasing the use of a particular antibiotic will select against resistant strains (9; 135). This finding supports the hypothesis that compensatory evolution acts to lessen the frequency of reversion by increasing microbial fitness in the absence of antibiotic pressure (9).

Figure 4. Modeling of the relationship between antibiotic resistance and fitness.

      In the traditional view of antibiotic resistance and fitness, resistant bacteria are selected in the presence of an antibiotic, regardless of whether they are more or less fit. Antibiotic resistance often leads to a fitness defect; however, in the absence of the antibiotic, resistant bacteria can persist in a population, possibly through compensatory mutation. Sensitive bacteria are annotated by (S). Resistant bacteria are annotated by (R). Compensatory mutants are annotated by (R_c) (A). Laboratory modeling of this relationship is measured through competition assays, both *in vitro* and *in vivo,* where either a flask or animal is inoculated with a 50:50 mixture of resistant and sensitive bacteria, and then cultured over time on selective and non-selective media (B).
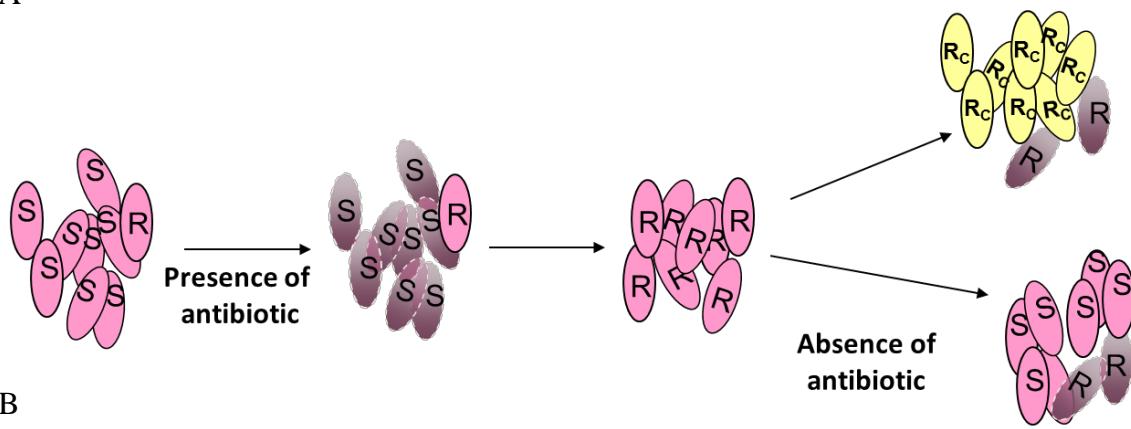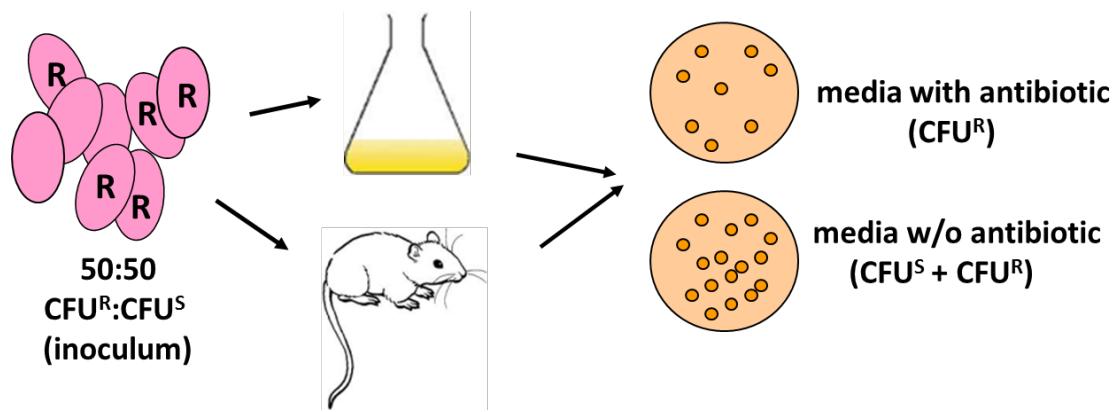
1.6a β-lactam Resistance in Other Organisms

Studies of β-lactam resistance point to a link between mechanisms of resistance and loss of fitness. *Streptococcus gordonii* strains with penicillin resistance due to PBP mutations have significantly longer generation times and form smaller colonies than the susceptible parent strain *in vitro.* Thirty-six passages of *S. gordonii* in the presence of sub-inhibitory levels of penicillin result in a loss of fitness. Over an additional seventy-nine passages, fitness increased. Fitness was not fully compensated when competed against wild-type strains. The mutations that led to the fitness increase had no effect on penicillin resistance, and were determined to be *in vitro*-selected compensatory mutations (59).

β-lactam resistance in *Streptococcus pneumoniae* is a result of mutations in the three PBPs involved in cell wall synthesis and cell division: PBP1a, PBP2x and PBP2b. Albarracín Orio *et al.* demonstrated both *in vitro* and in a murine model *in vivo*, that mutations to *pbp2b* confer a fitness cost to *S. pneumoniae* (4). Acquisition of certain secondary mutations in both *pbp2x* and *pbp1a* fully compensated this defect; these investigators identified this mechanism of compensation in a novel clinical isolate (4). Trzcínski *et al.* observed a continual decrease in fitness *in vivo* in penicillin-resistant strains of *S. pneumoniae* with increasing mutations in the PBPs in an infant rat model of colonization (156).

1.6b *Neisseria gonorrhoeae*

The effect of resistance determinants in Gc have been studied *in vitro* and *in vivo* for a select few antimicrobials. Warner *et al.* showed that mutations that inactivate or downregulate *mtrR* and thus, increase expression of *mtrCDE,* increase fitness *in vivo*

(170). This fitness benefit is likely a result of increased efflux of host effectors of the innate immune response, such as antimicrobial peptides at the site of infection. Several different clinically relevant *mtr* locus mutations can de-repress *mtrCDE*, and later work by Warner *et al.* demonstrated a relationship between the level of antimicrobial resistance conferred by different *mtr* locus mutations and the resultant *in vitro* and *in vivo* fitness of the bacterium (171). Kunz *et al.* demonstrated that mutations to *gyrA*, which confer intermediate level fluoroquinolone resistance to Gc, confer a fitness advantage *in vivo*. Addition of the *parC* mutation nullified this fitness gain (84). Changes in gene transcription as a result of the *gyrA* mutation are the likely cause of the observed fitness benefit (37).

1.7 TRANSFER OF RESISTANCE DETERMINANTS

Horizontal gene transfer is critical to the spread of antibiotic resistance. In 1966, in the M.C.R. Industrial Injuries and Burns Unit at the Birmingham Accident Hospital in England, carbenicillin was introduced to treat burn infections (128). Three years later, resistance began to appear in the form of highly resistant carbenicillinase-producing wound isolates. Evidence indicates that the resistance spread from a resistant strain of *Pseudomonas aeruginosa* to a susceptible strain within the same wound. Roe *et al.* used a mouse burn model of infection to demonstrate that the R-factor encoding carbenicillinase was transferrable to a susceptible strain of *P. aeruginosa* and also to *E. coli* and other Gram-negative organisms in a mixed infection of the same burn (128).

Horizontal gene transfer via conjugation or transposition, can also occur between Gram-positive and Gram-negative bacteria (34). Evidence suggests that this transfer is polar, from reservoirs of Gram-positive cocci to Gram-negative pathogenic bacteria.

Horizontal gene transfer from Gram-positive to Gram-negative bacteria has been demonstrated *in vitro*, and is inferred to occur naturally from *Streptococcus sp., Staphylococcus sp.* or *Enterococcus sp.* to *Campylobacter* spp., *E. coli, Klebsiella pneumoniae,* and some *Neisseria* spp. for several resistance determinants (34).

Enterococci, present in the human bowels, contain conjugative plasmids and transposons (38). Enterococcal resistance to glycopeptide antibiotics results from the acquisition of the *vanA* or *vanB* gene. High-level vancomycin and teichoplanin resistance is conferred by *vanA*, while *vanB* encodes vancomycin resistance. Dahl *et al.* demonstrated that *in vitro* transfer of *van* could be reproduced *in vivo,* and that the rate of transfer was increased *in vivo* (38). The large number of exconjugants present and the stability of these resistance mutations in the absence of antibiotic pressure suggest that this transfer can occur between transient colonizers and commensal flora, when no chemotherapy is in use (38).

1.7a *Neisseria gonorrhoeae*

In 1974, the spread of antibiotic resistance in Gc was already a concern. It was unknown at the time whether resistance spread via plasmid or through natural transformation. Sarubbi *et al.* postulated that plasmid-mediated antibiotic resistance would spread from cell-to-cell during mixed cultures of sensitive and resistant strains of gonococci, and that the resistance may be plasmid-encoded, like the R-factors of enteric bacteria (134). They examined spectinomycin, streptomycin, erythromycin, and rifampin resistance. They determined that the transfer of resistance was blocked by DNase I, and was likely the result of successful transformation of the susceptible strain with exogenous DNA released from the donor strain into the culture media (134). These early studies

showed that resistance could spread from strain to strain. However, it was not entirely

dependent upon the transfer of plasmids, as the researchers had hypothesized.

The source of DNA for natural transformation can be DNA released by autolysis

of cells and also DNA found within outer membrane vesicles (OMVs). Naturally

occurring membrane blebs, or OMVs, are detected in Gc cultures. In 1989, Dorward *et

al.* examined these blebs and found that the OMV fractions contained genetic material,

including RNA, linear DNA, and circular DNA. They recognized that these OMVs may

be a route for the transfer of genetic resistance, and in *in vitro* experiments, determined

that penicillinase-producing R-plasmids could be transferred during incubation of OMVs

with a penicillin-sensitive population, thus documenting shedding of OMVs as a possible

mechanism for intercellular transfer of resistance determinants *in vivo* (40).

However, as in the following example of tetracycline resistance, later research

demonstrated that plasmids are also a vital source of interspecies spread of resistance.

High-level resistance to tetracycline is conferred by *tetM*, a resistance determinant

located on a conjugative transposon that was originally identified in *Streptococcus* (104).

In the early 1980s, identification of this plasmid in *Mycoplasma hominis, Ureaplasma

urealyticum,* and *Gardnerella vaginalis,* all residents of the genital tract, led to concern

that this resistant determinant might be the cause of high-level tetracycline resistance in

Gc. Using a set of 79 tetracycline-resistant strains, Morse *et al.* discovered that all cases

of high-level tetracycline resistance were not due to chromosomally encoded resistance,

but to the presence of the *tetM* gene on a 25.2 mega Dalton (MDa) conjugative plasmid.

Evidence supported the theory that this resistance gene was introduced into the plasmid

through a one-time transfer event, and the plasmid then disseminated to other Gc strains (104).

A recent example of transformation of resistant alleles leading to clinically relevant resistance is the acquisition of mosaic penA alleles from commensal *Neisseria* spp. In 1992, Spratt *et al.* identified *penA* alleles involved in penicillin resistance as having regions that more closely resembled DNA from commensals *N. flavescens* and *N. cinerea* (148). These alleles were called mosaic *penA* alleles, and transformation was identified as the mechanism of interspecies genetic transfer (148). Further studies determined that commensal species *N. perflava, N. sicca, N. cinerea,* and *N. flavescens* all contributed DNA that resulted in the mosaic alleles, presumably through recombination following uptake by natural transformation (5; 154; 179).

1.8 STATEMENT OF PROBLEM

Approximately 106 million cases of gonorrhea occur worldwide each year (108). Gonorrhea significantly affects reproductive health and increases transmission of HIV (70; 75; 110; 168). Antibiotic treatment is a critical control measure; however, recently Gc has been elevated to "superbug" status as a result of the emergence of ceftriaxone-resistant strains, which raises the specter of untreatable gonorrhea (162). Ceftriaxone resistance in Gc is conferred primarily by mosaic *penA* alleles that encode an altered PBP2 (155). Whether acquisition of these mosaic alleles is accompanied by a fitness cost is unknown. Will the use of ceftriaxone/azithromycin dual therapy stop the spread of multi-drug resistant Gc? The likely answer, based upon the history of this organism and antibiotic use, is no.

More research is needed to understand what fitness cost these mosaic *penA* resistance alleles confer on the bacterium. Based on the importance of PBP2 in peptidoglycan synthesis, we hypothesize that mosaic *penA* alleles that confer high-level ESC-resistance decrease fitness in Gc, but that compensatory mutations arise to alleviate these fitness defect(s), which can lead to persistence and spread of resistant strains.

An increased understanding of the role of horizontal gene transfer between strains of Gc during infection is also critical to understanding the spread of chromosomal resistance determinants. While the transfer of chromosomal resistance has been demonstrated *in vitro*, with the exception of a rhesus macaque study in which horizontal transfer of rifampin and streptomycin resistance between two different strains of *Neisseria macaque* was demonstrated(174), the transfer of chromosomal resistance determinants between pathogenic strains of *N. gonorrhoeae* has never been demonstrated in an *in vivo* model.

We hypothesize that resistance determinants that confer fitness benefits to Gc (i.e. $mtrR_{-79}$, $gyrA_{91/95}$) are more easily transferred during infection due to host-mediated selection of the transformants. Those alleles that confer a fitness cost (i.e. mosaic *penA* alleles) are less likely to be selected for during infection due to the high biological cost associated with these alleles. If data in support of this hypothesis are obtained, these results might explain in part why we have yet to see the spread of ceftriaxone-resistance gain traction worldwide.

1.9 SPECIFIC AIMS

Aim 1: Determine the fitness cost or benefit of the *penA41* and *penA89* mutant alleles in *Neisseria gonorrhoeae in vitro* and *in vivo*

Hypothesis:

Mosaic *penA* alleles that change the structure of PBP 2 (*penA41, penA89*) will confer a fitness cost *in vitro* and *in vivo*, but compensatory mutations may be selected that restore fitness without reducing antibiotic resistance.

Aim 2: Determine whether genetic transfer of resistance alleles *penA41, mtrR$_{-79}$,* or *gyrA$_{91/95}$* occurs during co-infection of resistant (donor) and susceptible (recipient) Gc strains.

Hypothesis:

Mixed Gc infections may lead to the spread of antibiotic resistance, and resistance determinants known to increase bacterial fitness *in vivo* (*gyrA$_{91/95}$*, *mtrR$_{-79}$*) will be successfully transferred more frequently *in vivo* to susceptible strains than resistance determinants that decrease fitness (*penA41*).

CHAPTER 2: Materials and Methods

2.1 STRAINS AND PLASMIDS

2.1a Fitness Studies

Descriptions of the bacterial strains and oligonucleotide primers used in fitness studies are shown in Tables 1 and 2. The antibiotic susceptible laboratory strain FA19, which was first isolated in 1959 (94), was used as the background strain for all fitness studies. As streptomycin resistance is required for mouse infection studies, the *rpsL* gene from FA1090 was introduced into strain FA19 to create strain FA19 *rpsL* by spot transformation (58). The *rpsL* gene encodes ribosomal protein S12. Strain FA1090 carries a mutation *rpsL,* which confers streptomycin resistance (146).

Strains H041 and F89, both high-level ceftriaxone-resistance clinical isolates, were obtained from Dr. Magnus Unemo (World Health Organization Collaborating Centre for Gonorrhea and other Sexually Transmitted Infections; Swedish Reference Library for Pathogenic Neisseria; Örebro University Hospital) (116; 158). Strains FA19 *penA41* and FA19 *penA89* were provided by Dr. Robert Nicholas (University of North Carolina) and are mutants of FA19 *rpsL* in which the wild-type *penA* gene was replaced by the mosaic *penA* alleles from Gc strains H041 and F89, respectively, by allelic exchange. Strain FA19 *penA41*, *cat* was constructed by introducing a ClaI fragment carrying a chloramphenicol acetyltransferase (*cat*) gene from the *Neisseria* Chromosomal Integration Vector pGCC5 (Figure 5) into a nonessential intergenic region in mutant FA19 *penA41* (101). Strains LV41A, LV41B, LV41C, and LV41E are mouse vaginal isolates from *in vivo* competitive infections with strains FA19 *rpsL* and FA19 *penA41*. The *acnB*$_{G348D}$ mutation from strain LV41C was introduced into strains FA19 *penA41,* FA19 *rpsL,* and FA19 *penA89* in Dr. Robert Nicholas' laboratory to create strains FA19 *penA41, acnB*$_{G348D}$, FA19 *rpsL, acnB*$_{G348D}$, and FA19 *penA89, acnB*$_{G348D}$, respectively. The construct

carrying the $acnB_{G348D}$ allele contained a silent NotI digestion site near the site of the mutation, a kanamycin resistance cassette at the 3' end, and was made with and without a histidine tag (Figure 5). Transformants were selected on GC agar plates containing 50 µg/mL kanamycin. All Gc strains were propagated on solid GC agar containing Kellogg's supplement I (79) and 12 µM ferric nitrate for 18-20 hours at 37° C in 7% CO_2. Passage from frozen stocks was minimized to reduce the risk of acquiring secondary mutations.

Table 1. *N. gonorrhoeae* strains used in fitness studies

Strain	Description	Reference
FA19	Antibiotic-sensitive cervical isolate from a patient with disseminated gonococcal infection (Denmark, 1959)	(94)
FA19 *rpsL*	FA19 carrying the *rpsL* gene from strain FA1090; Sm^R	This study
H041	Pharyngeal isolate (Japan, 2009); Cro^R, Cfx^R, Pen^R, Cip^R, Azm^R, Tet^R, Spc^S	(116)
F89	Male urethral isolate (France, 2010); Cro^R, Cfx^R, Pen^I, Cip^R, Azm^R, Tet^R, Spc^S	(158)
FA1090	Cervical isolate from a case of disseminated gonococcal infection; Sm^R	(146)
FA19 *penA41*	FA19 *rpsL*, *penA41*; Sm^R, Cro^R	This study
FA19 *penA89*	FA19 *rpsL*, *penA89*; Sm^R, Cro^R	This study
FA19 *penA41, cat*	FA19 *penA41*, *cat;* Sm^R, Cm^R	This study
LV41A	Vaginal isolate from competitive murine infection with FA19 and FA19 *penA41*; Sm^R, Cro^R	This study
LV41B	Vaginal isolate from competitive murine infection with FA19 and FA19 *penA41;* Sm^R, Cro^R	This study
LV41C	Vaginal isolate from competitive murine infection with FA19 and FA19 *penA41;* Sm^R, Cro^R	This study
LV41E	Vaginal isolate from competitive murine infection of FA19 and FA19 *penA41;* Sm^R, Cro^R	This study
FA19 *penA41*, *acnB*$_{G348D}$	FA19 *penA41, acnB*$_{G348D,}$ *kat*::*acnB;* Sm^R, Cro^R	This study
FA19 *penA89*, *acnB*$_{G348D}$	FA19*penA89, acnB*$_{G348D,}$ *kat*::*acnB;* Sm^R, Cro^R	This study

Table 2. Oligonucleotide primers used in this study

Primer	Nucleotide Sequence
*rps*LF	5'-GCCGTCTGA AAT GCTTGACTGTCTGCTTGC-3'
*rps*LR	5'-GCTGGGTCTATTCCCATGAA-3'
*pen*AF1	5'-CGGAATCGGATTCCTGCA T-3'
*pen*AF2	5'-CTGATGGTATTCGACCGC-3'
*pen*AR1	5'-CCTCGATCAGAGGATTCAGAC-3'
*pen*AR2	5'-GCGAATCTGCCGTCGT-3'
*rec*AF1	5'-CAACTTATATCGTATGGGGCTGAC-3'
*rec*AR1	5'-GCCGTCTGAAATCACCGATGGGGAAGATCG-3'
*rec*AF2	5'-ATGTCAGACGACAAAAGCAAAGC-3'
*rec*AR2	5'-GCCAACGACCCGAAGAAT-3'

Figure 5. Vectors and DNA constructs used for strain construction.

      (A) The *Neisseria* Chromosomal Integration Vector pGCC5 (NICS; a gift of H. S. Seifert, Northwestern University) contains a *cat* resistance cassette between the *lctP* and *aspC* genes with two ClaI restriction sites available to cut out the plasmid backbone (figure from H.S. Seifert, Northwestern University (unpublished work). (B) The genomic DNA organization around the *acnB* gene. (C) The construct used to create strains FA19 *penA41, acnB*$_{G348D}$, FA19 *rpsL, acnB*$_{G348D}$, and FA19 *penA89, acnB*$_{G348D}$. (Designed by R.A. Nicholas and I. McDonald, University of North Carolina, Chapel Hill)

A
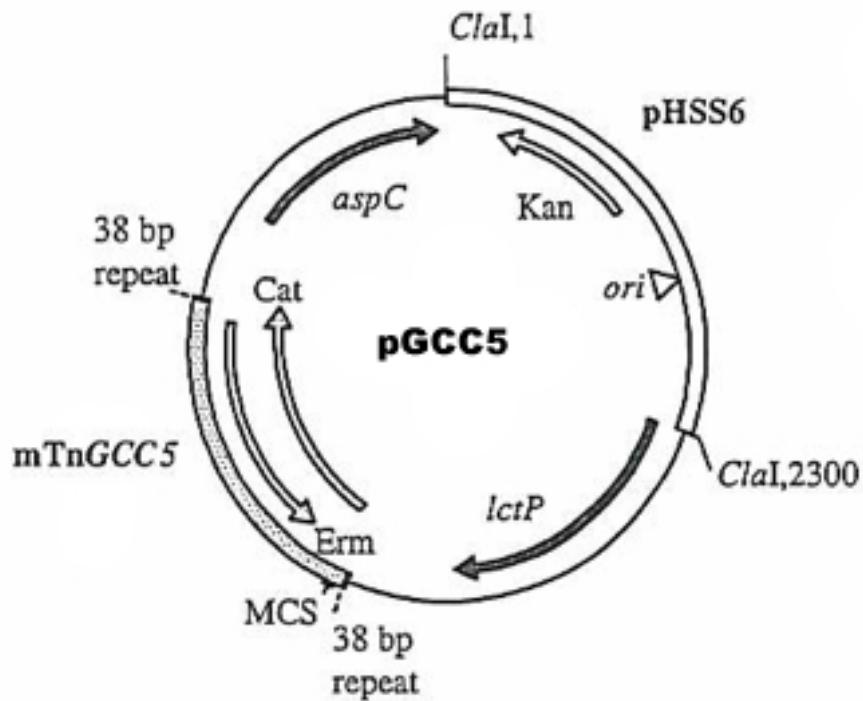
B
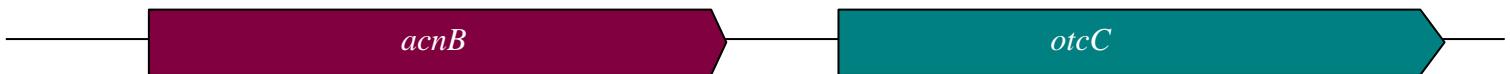
C
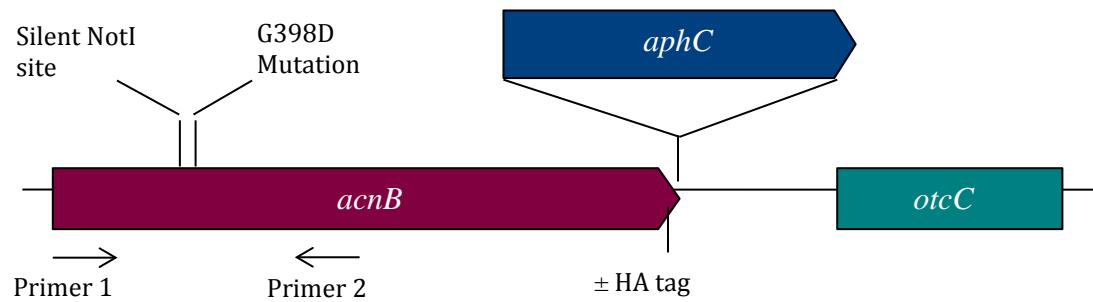
2.1b Transfer of Antibiotic Resistance

A description of the strains used in transfer of antibiotic resistance studies is shown in Table 3. *Escherichia coli* strain E74-00 carries an insertionally inactivated Gc *recA* gene (*recA*::*aph*A-3) cloned into pACYC184 (144). E74-00 was harvested from overnight growth in L-broth with 50 μg/mL kanamycin. Plasmid mini-preparations were conducted using 1 mL of culture and the Qiagen Qiaprep Miniprep kit (Qiagen #27106), and the *recA*::*aphA-3* gene was PCR-amplified as described below and transformed into Gc strains AK1, KH15 and FA19 *penA41* to create strains AK1 *recA⁻*, KH15 *recA⁻*, and FA19 *penA41, recA⁻* (84; 171).

Table 3. Bacterial strains used in transfer of resistance studies

Strain	Genotype	Source
OP100	FA1090, *cat;* Cm^R	(141)
E74-00	*E. coli* HB101 carrying pACYC-184 with *recA*::*aph*A-3 inserted into the multicloning site	(102; 143; 144)
AK1	FA19Sm^R *gyrA$_{91/95}$*	(84)
KH15	FA19Sm^R *mtrR*	(171)
FA19 *penA41*	FA19 *rpsL*, *penA41*	This study

2.1c Molecular Biology Methods

The primers used to amplify *rpsL* (*rpsL*F and *rpsL*R) carried the *Neisseria* DUS (44; 77). The conditions of the polymerase chain reaction (PCR) were 10 minutes at 94°C for one cycle, 30 cycles of 30 seconds at 94°C, 30 seconds at 53°C, 1 minute at 72°C followed by one cycle of 10 minutes at 72°C. The *recA::aph*A-3 gene was PCR-amplified using PCR primers *recA*F1 and *recA*R1 (Table 2). PCR conditions were 2 minutes at 94°C for one cycle, 30 cycles of 30 seconds at 94°C, 1 minute at 54°C, 2 minutes at 72°C followed by one cycle of 5 minutes at 72°C. To verify strain construction, *recA::aph*A-3 was amplified from mutant strains using primers *recA*F1 and *recA*R1 (Table 2) (141). The PCR program used for verification of all strains was 10 minutes at 94°C for one cycle, 30 cycles of 30 seconds at 94°C, 1 minute at 55°C, 2 minutes at 72°C followed by one cycle of 10 minutes at 72°C. For all DNA transformations, PCR products were purified using a 2% agarose gel run in 1X TAE buffer and extracted using the Qiagen QIAquick gel extraction kit (Qiagen # 28704).

Confirmation of all mutations was by PCR amplification and nucleotide sequence analysis. Nucleotide sequencing was done through the USUHS Biomedical Instrumentation Center (BIC) using the ABI 3500xL Genetic Analyzer. Samples were first purified according to the manufacturers protocol using the Qiagen QIAquick PCR Purification kit (Qiagen #28104), followed by the sequencing reaction utilizing ThermoFisher Scientific BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems # 4337455). Genomic DNA was extracted using the Promega Wizard Genomic DNA purification kit (Promega #A1120) according to the manufacturer's protocol. For DNA transfer experiments, the extracted DNA was rehydrated overnight, and the DNA was quantified using the NanoDrop 1000 spectrophotometer.

2.2 BACTERIAL CULTURE CONDITIONS AND ANALYSIS OF *IN VITRO* GROWTH

2.2a Growth Curves

To analyze *in vitro* growth kinetics, bacterial colonies with non-piliated morphology were harvested with a sterile swab and inoculated into supplemented Gc broth with 5mM $NaHCO_3$ (84). Cultures were shaken at 220 RPM at 37°C, and bacterial growth was assessed by measuring the OD_{600} at hourly intervals for a total of eight hours. Aliquots were also quantitatively cultured every two hours to determine the number of colony forming units per milliliter (CFU/mL). Experiments were repeated six to twelve times, and data were combined and analyzed by a two-way analysis of variance (ANOVA). Differences in growth rate were measured by comparing the average number of minutes it took to reach an OD_{600} of 0.8 for each strain as described (84). Results were compared using a one-way analysis of variance to determine overall significance followed by a student's *t*-test to determine significance between individual strains.

2.2.b Competitive Co-cultures

Bacterial strains to be competed were harvested from solid GC agar plates and suspended in GC broth. Bacterial suspensions were then adjusted to an OD_{600} of 0.8 and equal volumes of each strain to be compared were inoculated into supplemented GC broth with 5mM $NaHCO_3$. Cultures were shaken at 220 RPM at 37°C. Aliquots were taken at time 0 and every two hours, and quantitatively cultured on selective (mutant CFU) and non-selective media (total CFU). Ceftriaxone (0.125 µg/mL) was used to select for FA19 *penA41,* LV41A, LV41B, LV41C, and LV41E; 0.008 µg/mL of ceftriaxone was used to select for FA19 *penA89*. Chloramphenicol (0.5 µg/mL) was used to select for FA19 *penA41*, *cat* and kanamycin (50 µg/mL) was used to select for FA19 *penA41, acnB*$_{G438D}$ and FA19 *penA89, acnB*$_{G348D.}$ A competitive index (CI) was determined at each time point, whereby the ratio of mutant to wild-type bacteria at output for

each individual time point was divided by the ratio of mutant to wild-type bacteria at input (176). Experiments were conducted three times, and the geometric mean CI was used to determine fitness.

2.2.c *In vitro* Fitness Under Iron Limitation

To test the effect of iron limitation on the growth of strains carrying the $acnB_{G348D}$ allele, bacteria were inoculated into 4 mL of GC broth with Kellogg's supplements I, II, and 5mM $NaHCO_3$, and grown to mid-log phase. Samples were taken in 1 mL aliquots and spun for 2 minutes at 13,000 RPM in the microcentrifuge (Eppendorf centrifuge 5415 R), and the supernatants were removed. Samples were resuspended in GC broth, and the OD_{600} was adjusted to 0.8. Samples were then inoculated into 35 mL of GC broth with Kellogg's supplement I, 5 mM $NaHCO_{3,}$ and 50 μM deferoxamine mesylate (DFM) (Sigma #D-9533), an iron chelator (72). Samples were incubated with shaking at 220 RPM at 37°C for six hours. The OD_{600} was measured every hour, and aliquots were cultured after zero, two, four and six hours of incubation on GC agar.

For competitive co-culture in iron-depleted conditions, bacteria were inoculated into 4 mL of GC broth with Kellogg's supplements I, II, and 5 mM $NaHCO_3$ and grown to mid-log phase. Samples were taken in 1 mL aliquots and spun for 2 minutes at 13,000 RPM in the microcentrifuge (Eppendorf centrifuge 5415 R), and the supernatant was removed. Samples were resuspended and adjusted to an OD_{600} of 0.8, and equal volumes of each strain to be compared were inoculated into GC broth supplemented with Kellogg's supplement I, 5mM $NaHCO_{3,}$ and 50 μM DFM. Samples were incubated with shaking at 220 RPM at 37°C for six hours. Aliquots were cultured after zero, two, four and six hours of incubation on GC agar and selective GC agar containing either chloramphenicol (0.5 μg/ml) or ceftriaxone (0.125 μg/ml).

2.2d Phase Contrast Microscopy

Bacteria grown to mid-log phase (OD_{600} of 0.5-0.8) were suspended in GC broth

containing 0.05% saponin. Cells were visualized with an Olympus BX50 light microscope with

100x phase contrast and imaged with an Olympus DP71 microscope digital camera.

2.2e Scanning Electron Microscopy

Scanning electron microscopy was performed as described previously (107). In short,

strains FA19, FA19 *penA41,* and LV41A were grown to mid-log phase in supplemented liquid

growth medium, and 100 μL of each culture were centrifuged. The bacterial pellet was

resuspended in 500 μL of 3% glutaraldehyde and 0.15 M sodium phosphate buffer (pH 7.4) and

fixed at room temperature for thirty minutes. Fixed samples were applied to poly-L-lysine-coated

coverslips and examined using a Zeiss Supra 25 field Emission Scanning Electron Microscope at

5 kV, with a 5-mm working distance and a 10-μm aperture (Carl Zeiss SMT Inc., Peabody, MA).

2.2f Peptidoglycan Analysis

Bacteria were grown to an OD_{600} of 0.6 in 500 mL of supplemented Gc broth. The culture

was rapidly chilled in an ice-alcohol bath and centrifuged at 8000 x g. The resultant cell pellet

was washed with ice-cold 20 mM sodium acetate and resuspended in 10 mL of the same buffer.

The suspension was added dropwise to 10 mL of boiling 4% SDS, boiled for 30 minutes, and left

to cool at room temperature overnight. On day two, suspensions were subjected to

ultracentrifugation at 100,000 x g for 30 minutes (Sorval WX Ultra 80 Series), and the pellet was

washed five times with warm distilled water as described by Antignac *et al.* (10). The pellet was

resuspended in 5 mL 100 mM Tris-HCl and 10 mM NaCl. The suspension was subsequently

incubated with 100 μg of α-amylase (Sigma-Aldrich #10069) in 1 mL of 10 mM Tris-HCl buffer

at pH 7.0 for two hours at 37°C to degrade high molecular weight glycogen. Bound lipoproteins

were digested with 100 µg/mL of pronase (Sigma-Aldrich # P6911) for 90 minutes at 60°C. SDS-insoluble material was extracted in 1% SDS for 15 minutes and left to cool overnight. Samples were collected by centrifugation as described above and washed four times with warm water. Samples were resuspended in 0.5 mL of 20 mM sodium phosphate buffer (pH 4.8) and digested with 20 µg/mL muramidase lysozyme (Sigma-Aldrich #L6876) at 37°C overnight. The lysozyme reaction was stopped by boiling samples for 4 minutes. Insoluble material was removed by centrifugation for 5 minutes in the Eppendorf centrifuge. Finally, to prepare the samples for HPLC, 100 µL of the muropeptide solution were added to 100 µL of 0.5 M sodium borate, pH 9.0. One to two milligrams of sodium borohydride were added, and samples were incubated for 30 minutes at room temperature. Excess sodium borohydride was inactivated by adding 20% phosphoric acid (10).

Reverse-phase high performance liquid chromatography (HPLC) analysis was performed as described previously (10). In brief, a linear gradient from 0-15% of HPLC-grade water with 0.1% trifluoroacetic acid to acetonitrile was applied for 70 minutes to a column (4.6 x 250 nm; 5 µm particles) at room temperature with a 0.6 mL/min flow rate. UV peaks were detected at 210 nm. A 50 µL injection volume of peptidoglycan sample was used.

2.3 ANTIMICROBIAL SUSCEPTIBILITY TESTING

The MICs of ceftriaxone, penicillin, erythromycin, ciprofloxacin and chloramphenicol were determined according to the agar dilution method established by the Centers for Disease Control and Prevention (25). In brief, GC agar containing two-fold decreasing concentrations of each antibiotic tested was prepared in six-well tissue culture plates. Bacteria were grown overnight on solid Gc media and then suspended in 4 mL of phosphate buffered solution (PBS) to an A_{600} of 0.05. Ten microliters of the suspended bacteria were inoculated onto agar at each

antibiotic concentration. The plates were incubated overnight at 37°C, and results were read at 24- and 48-hours post-inoculation. The MIC was the lowest concentration of antibiotic from which no CFU were recovered.

2.4 COMPETITIVE MURINE INFECTION

Female BALB/c mice (4-6 weeks old) were treated subcutaneously with 0.5 mg of either water-soluble β-estradiol (Sigma-Aldrich #E-4389), when competing FA19 *rpsL* against recombinant strains FA19 *penA41* and FA19 *penA89,* or oil-soluble β-estradiol 3-benzoate (Sigma-Aldrich #E-8515) suspended in sesame oil (Sigma-Aldrich #S-3547), when competing compensatory mutants against parent and wild-type strains. In all experiments, estradiol was given on days -2, 0 and +2 of infection. Mice were also treated with streptomycin sulfate and vancomycin by intraperitoneal injection, and given trimethoprim (0.4g/L of drinking water) to promote gonococcal infection in accordance with previously published protocols (73; 84). Two days after the first injection of estradiol, mice were inoculated intravaginally with 20 µL of a mixed bacterial suspension containing 1-2 x 10^6 CFU of each strain (n = 4 –8 mice per experiment). Vaginal swabs were collected on days 1, 3, 5, and 7 post-inoculation and suspended in 1 mL of PBS. Equal volumes of vaginal swab suspensions were quantitatively cultured on GC agar with streptomycin (100 µg/ml) (total CFU) and GC agar with streptomycin and 0.125 µg/ml ceftriaxone (mutant CFU). A competitive index (CI) was determined whereby the ratio of mutant to wild-type bacteria at output for each individual time point was divided by the ratio of mutant to wild-type bacteria at input, as described elsewhere (176). Experiments were conducted two or three times for each mixture.

2.5 HIGH-THROUGHPUT SEQUENCING AND GENOME ASSEMBLY

A mate-pair library was constructed using Nextera XT standard kits (Illumina FC-121-1024 and FC-121-1001) and prepared for emulsion-based amplification and paired-end sequencing. Illumina MiSeq 2500 Genomic DNA paired-end sequencing was conducted by the Tufts University Core Facility using a v2 flow cell paired-end 250 base format. Each read was exactly 251 bases with two reads per insert plus a barcode. The insert for PE250 with Nextera XT ranges from 500 bp to 1300 bp, with the average/peak size range around 800 bp. BWA ALN and BWA SAMPE v0.5.9-r16 (90) were used to align the paired end sequence reads against the reference *Neisseria gonorrhoeae* FA19 scaffolds (NZ_ABZJ0000000; downloaded 07/08/14 (1)) with duplicates removed and alignment statistics generated with Picard v.1.48 (21). Coverage levels and polymorphisms were examined with local Perl and shell scripts applied to the output of SAMTOOLS MPILEUP v.0.1.19 (89; 91). The sequences of specific regions were identified using BCFTOOLS VIEW (88) on the MPILEUP output with FASTA files generated with the VCF2FA function in VCFUTILS (89). Data were re-analyzed using CLC Genomics Workbench version 6.0.4 against the recently sequenced FA19 genome sequence for verification (3).

2.6 GLUCOSE UTILIZATION

Glucose utilization by strains FA19 *rpsL,* FA19 *penA41,* LV41C, and FA19 *penA41, acnB*$_{G348D}$ was measured using the Roche-Biopharm kit for enzymatic bioanalysis of D-Glucose (Catalog #10716251035). The assay was modified for use in a 96-well plate. In brief, strains were inoculated in supplemented GC broth with 5mM $NaHCO_3$ and incubated for eight hours at 37°C, as described previously. The OD_{600} values of the cultures were recorded every two hours, and aliquots were cultured quantitatively to determine the number of CFU/mL. Concurrently, 1 mL samples were taken and centrifuged at 13,000 RPM for 4 minutes. The supernatants were passed through a 0.2 μm filter, and 10 μL of sample, 198.7 μL of distilled water and 99.3 μL of

Solution I were combined a mixed in a 96 well microtiter plate. The absorbance was recorded

(A_1) after three minutes using the CLARIOStar plate reader with a spectrometer (BMG Labtech).

Data were analyzed using the MARS Data Analysis Software package (Program Version 3.10

R5). The reaction was then started by the addition of two µL of Solution 2 (total volume 300 µl

per well), and the absorbance was read after 15 minutes (A_2). Results are expressed as the change

in absorbance (A_2-A_1=A_{340}), which indicates the amount of NADPH present at each time point,

divided by the A_{360}, which represents the absorbance of the total bacteria present at each time

point for any given strain.

2.7 TRANSFER OF ANTIBIOTIC RESISTANCE

2.7a Optimization of DNA Transfer

Transfer of resistance determinants to the antibiotic susceptible strain OP100 by natural

transformation of purified genomic DNA was optimized as follows. The source of the $gyrA_{91/95,}$

$mtrR_{-79}$, and $penA41$ resistance alleles were $recA$ mutants of strains AK1, KH15, and FA19

$penA41$, respectively (Table 3). Decreasing concentrations of genomic DNA (2.5 µg/mL, 1.25

µg/mL, 0.25 µg/mL, 125 ng/mL, and 50 ng/mL) from these strains were prepared in GC broth.

One half of each DNA suspension was treated with DNase I (Sigma #D5307)*;* incubation

conditions and the amount of DNase I added to each concentration of DNA were in accordance

with the manufacturer's instructions (134; 136; 145). Piliated colonies of the recipient strain

OP100 were inoculated into GC broth and grown to mid-log phase. DNA (10 µg, 5 µg, 1 µg, 500

ng, or 200 ng) with and without DNase I treatment was added to 1 mL of mid-log phase culture

and 3 mL of supplemented GC broth plus 5 mM $NaHCO_3$ for a total volume of 4 mL (final DNA

concentration was 2.5 µg/mL, 1.25 µg/mL, 0.25 µg/mL, 125 ng/mL, or 50 ng/mL, respectively).

Samples were incubated with shaking at 220 RPM at 37°C for six hours. Aliquots were cultured

at zero, four and six hours after inoculation on GC agar containing chloramphenicol (0.5 µg/mL) and ciprofloxacin (0.125 µg/mL), erythromycin (0.5 µg/mL), or ceftriaxone (0.125 µg/mL) to isolate OP100 transformants that expressed the desired resistance determinant from strains AK1 recA⁻, KH15 recA⁻, or FA19 penA41, recA⁻, respectively. Results were expressed as number of transformants/µg DNA.

2.7b Transfer of Resistance During Bacterial Co-culture

In order to determine whether resistance determinants are capable of being transferred between pathogenic strains of Gc *in vitro*, recombination-deficient donor strains and the OP100 recipient strain were co-cultured for ten hours. Aliquots were taken at zero, six, eight, and ten hours of culture and treated with DNase I for 15 minutes prior to being plated onto selective media. Selection for OP100 bacteria that had successfully acquired the mutations was performed using the methodology described above.

2.7c Transfer of Antibiotic Resistance *in vivo*

Female BALB/c mice (4-6 weeks old) were treated subcutaneously with 0.5 mg of oil-soluble β-estradiol 3-benzoate (Sigma-Aldrich #E-8515) suspended in sesame-oil (Sigma-Aldrich #S-3547) and antibiotics to promote Gc infection as described above. Two days after the first injection of estradiol, mice were inoculated intravaginally with 20 µL of a mixed bacterial suspension containing 1-2 x 10^6 CFU of OP100 and either AK1 recA⁻, KH15 recA⁻ or FA19 penA41, recA⁻. Vaginal swabs were collected every day for seven days post-inoculation and suspended in 500 µL of GC broth containing 0.05% saponin. Vaginal swab suspensions were treated with DNase I for 15 minutes and then cultured onto GC agar with streptomycin (total CFU) and GC agar with chloramphenicol and ciprofloxacin, erythromycin, or ceftriaxone as described for *in vitro* transfer experiments to isolate the desired transformants. Agar plates were

examined after 24- and 48-hours of incubation. The piliation phenotype based on colony morphology was recorded for the recipient strain, which was selected on plates with chloramphenicol and either ciprofloxacin, erythromycin, or ceftriaxone.

CHAPTER 3: Results

3.1 STUDIES ON THE EFFECT OF MOSAIC *PENA* ALLELES ON MICROBIAL FITNESS

3.1a Mosaic *penA41* and *penA89* Alleles Reduce *in vitro* Fitness

To assess the impact of mosaic *penA* alleles that confer ESC resistance on bacterial fitness, we introduced the *rpsL* mutation present in strain FA1090 to Cro^S strain FA19 to provide a Sm^R phenotype, which is necessary for experimental murine infection (74). We then transformed the mosaic *penA* alleles from strain H041 or F89 into strain FA19 *rpsL* as described in the Materials and Methods to make recombinant strains FA19 *penA41* or FA19 *penA89,* respectively (Table 1). As expected, the MICs of ceftriaxone for mutants FA19 *penA41* and FA19 *penA89* were 500-fold higher than that of the parent strain (Table 4). We hypothesized that changes in cell wall structure incurred by amino acid changes in PBP 2 (PenA*)* would impair bacterial growth. To test this hypothesis, strains FA19 *rpsL*, FA19 *penA41* and FA19 *penA89* were cultured independently in liquid media. Growth of the mutants was clearly impaired as evidenced by differences in both optical density (Figure 6A) and the number of viable bacteria recovered (Figure 6B) over time. The most pronounced fitness cost was observed for strain FA19 *penA89.* To examine further the consequence of these alleles on *in vitro* fitness, we performed co-cultures with the resistant mutants and the Cro^S parent strain. In three independent experiments, FA19 *penA41* bacteria were consistently less fit than the Cro^S parent strain FA19 *rpsL,* as evidenced by 10-fold and 50-fold decreases in the relative number of mutant gonococci recovered after six and eight hours of growth, respectively (Figure 7A, Table 5). As predicted from the noncompetitive cultures, we observed an even more pronounced fitness defect in mutant FA19 *penA89*, which when competed against FA19 *rpsL*, showed a 17-fold decrease in fitness within the first four hours of incubation and a 330-fold decrease in fitness at eight hours

(Figure 7B). We conclude that one or more mutations within each mosaic *penA* allele is

detrimental to Gc growth *in vitro*, with the *penA89* allele being the most attenuating.

Table 4. Minimum inhibitory concentration of antibiotics

Strain	Antibiotic Concentration (µg/mL)			
	Ceftriaxone	Penicillin	Ciprofloxacin	Erythromycin
FA19 *rpsL*	0.001	0.012	0.03	0.38
FA19 *penA41*	0.5	0.11	0.03	0.38
FA19 *penA89*	0.5	N/A	N/A	N/A
LV41A	0.5	0.11	0.03	0.38
LV41B	0.5	0.11	0.03	0.38
LV41C	0.5	0.09	0.03	0.38
LV41E	0.5	0.13	0.03	0.38

Figure 6. Mosaic *penA41* and *penA89* alleles negatively affected growth of the Cro^S wild-type
  parent strain.

  All strains were grown over an 8-hour period with agitation at 220 RPM and at 37°C. The optical density and CFU/mL were determined at 1-hour and 2-hour intervals, respectively. Results are combined mean data from three different independent experiments for each strain. Error bars show the standard error of the mean (SEM). Statistical significance was calculated using a 2-way ANOVA with multiple comparisons. *P*-value shown is for each mutant strain versus FA19 *rpsL* (A). *P*-values shown are for each mutant strain versus FA19 *rpsL* (B).

A
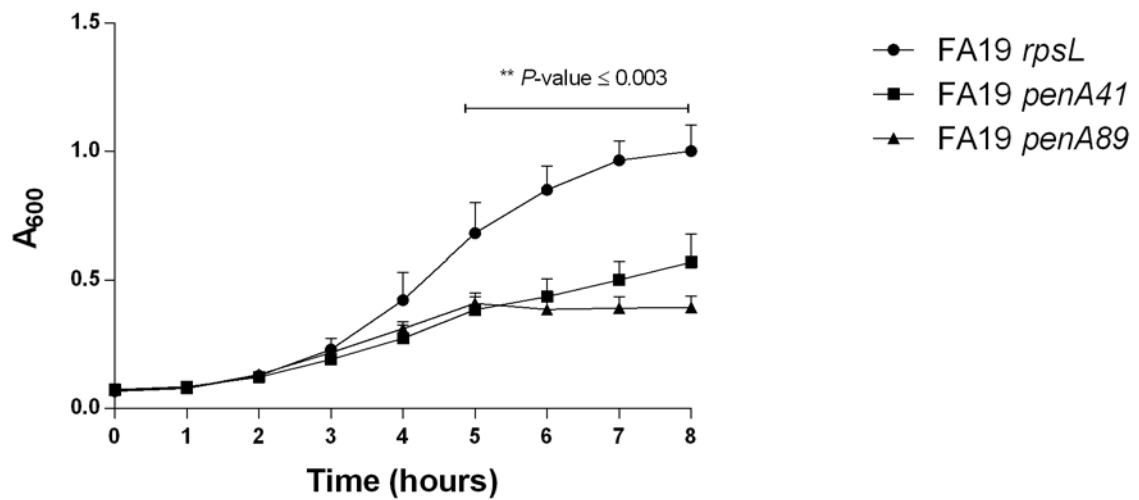
B
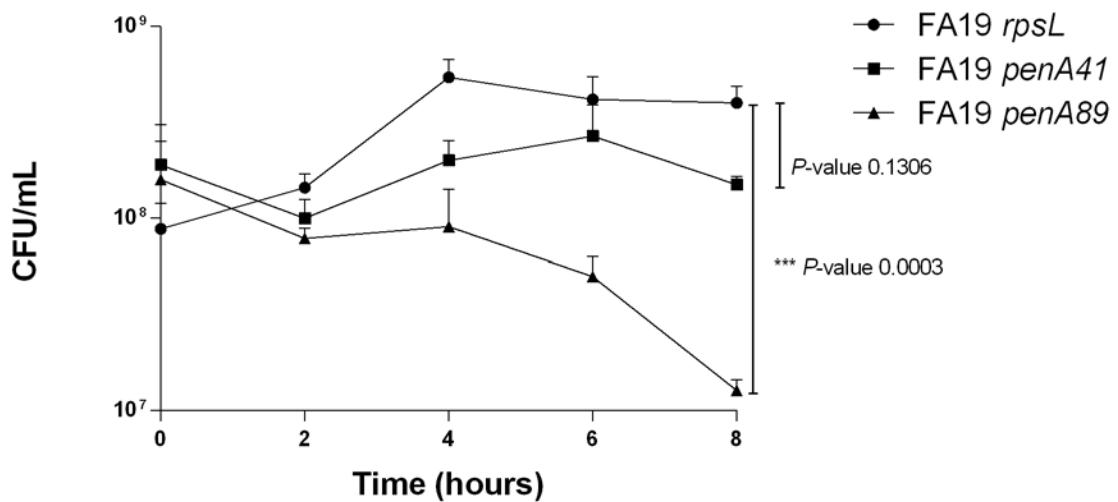
Figure 7. *In vitro* competition of mutant strains carrying mosaic *penA* alleles against the Cro[S] wild-type parent strain.

Competition index of wild-type FA19 *rpsL* competed against (A) FA19 *penA41* and (B) FA19 *penA89* grown in co-culture over an 8-hour time period. Similar numbers of each strain were inoculated into GC broth and the competition index was determined by dividing the ratio of mutant to wild-type bacteria at output by the ratio of mutant to wild-type bacteria at input. The horizontal bars indicate the geometric mean (also shown in parentheses), and the solid line indicates a competition index of one. Results are data from three independent experiments.

A

FA19 *rpsL* vs. FA19 *penA41*

B

FA19 *rpsL* vs. FA19 *penA89*
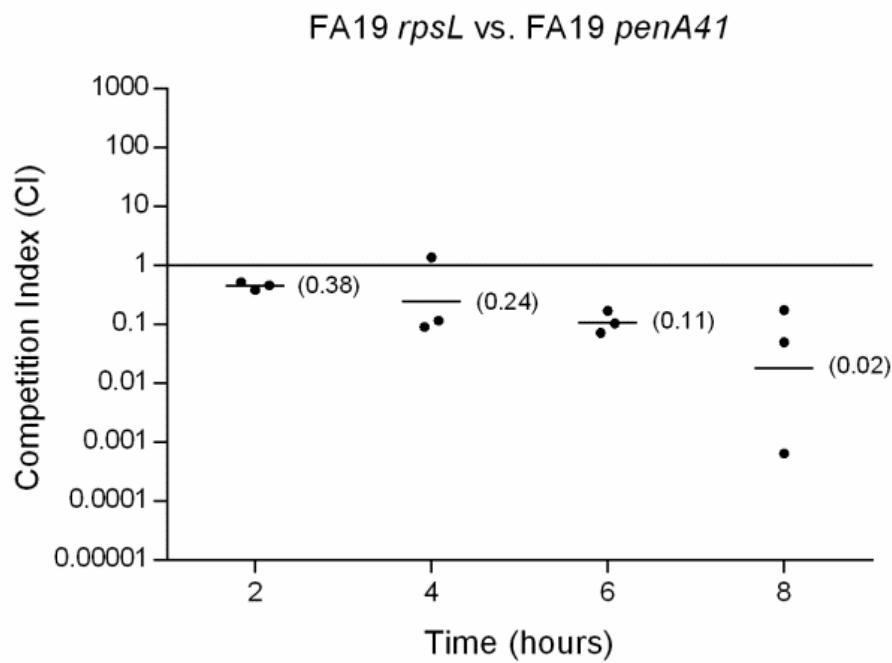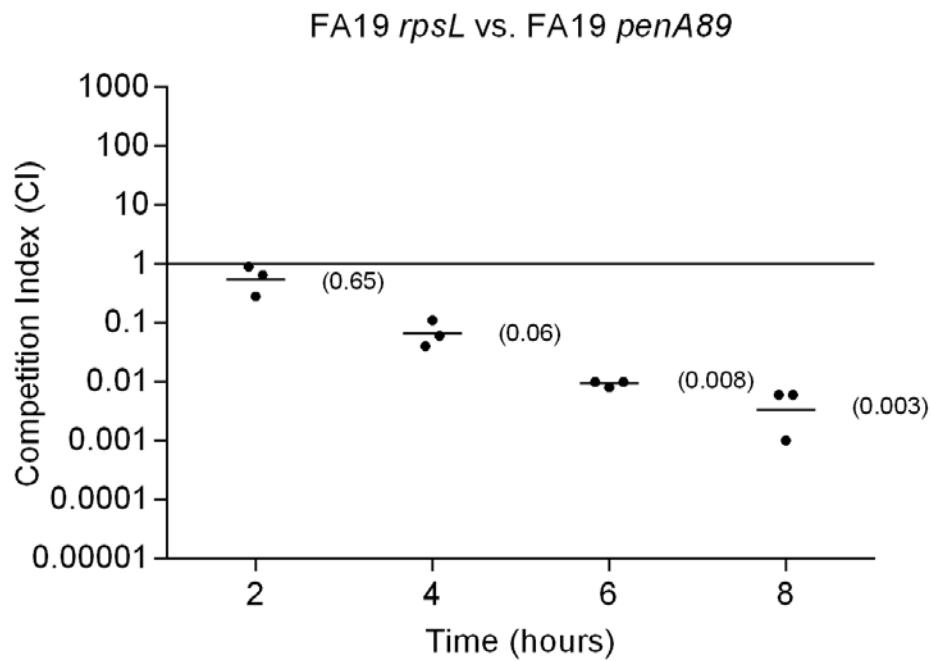
3.1b Mosaic *penA41* and *penA89* Alleles Reduce *in vivo* Fitness but Compensatory Mutants are Selected During Infection

To determine whether the fitness disadvantages observed *in vitro* also occur *in vivo*, we conducted a series of competitive infections in the murine model of gonococcal genital tract infection (73). BALB/c mice were inoculated vaginally with bacterial suspensions containing similar numbers of Cro^S (FA19 *rpsL*) and Cro^R bacteria (FA19 *penA41* or FA19 *penA89*), and the numbers of each strain isolated from vaginal mucus were determined on one, three, five and seven days post-inoculation. Overall, significantly fewer mutant bacteria were recovered from mice relative to the parent strain over the course of infection. A 14-fold decrease in fitness by day seven post-inoculation was observed for FA19 *penA41* (Figure 8A) and a 2 x 10^5-fold decrease in fitness was observed for strain FA19 *penA89* (Figure 8B). The more dramatic fitness cost conferred by the *penA89* allele was also evident by the recovery of no Cro^R FA19 *penA89* bacteria on days three, five or seven of infection as compared to high numbers of Cro^S bacteria (Figure 8B, open triangles). Interestingly, and in contrast to experiments with FA19 *penA89*, we detected an upwards shift in the CI in five of nine mice inoculated with FA19 *rpsL* and FA19 *penA41* after an earlier decrease (Figure 8A, CI values indicated by an X). In some cases, Cro^R bacteria completely out-competed the Cro^S strain (Figure 8A, circled X). We suspected that selection for compensatory mutations may have occurred in these mice and froze well-isolated colonies from the selective (Cro) agar plates. Four of these mutant strains, LV41A, LV41B, LV41C, and LV41E were subjected to further phenotypic and genotypic analyses. Mutant LV41D did not have a reproducible phenotype, most likely due to a heterogenous stock, and was not examined further.

Figure 8. *In vivo* competition of mutant strains carrying mosaic *penA* alleles against the Cro[S] parent strain.

Results of competitive murine infection with wild-type FA19 *rpsL* versus (A) FA19 *penA41* or (B) FA19 *penA89*. Mice were inoculated vaginally with similar numbers of each strain, and the competition index was determined by dividing the ratio of mutant to parent bacteria at output by the ratio of mutant to parent bacteria at input over a 7-day period. Each symbol indicates the CI for an individual mouse, and colors indicate separate experiments. The bars indicate the geometric mean. Open circles indicate that only mutant bacteria were recovered. Open triangles indicate that only wild-type bacteria were recovered. X's indicate CIs for mice from which putative compensatory mutants were first observed and X's within circles indicate mice from which only putative compensatory mutants were recovered. Isolates from these mice were saved for further analysis for potential compensatory mutation(s).

A

FA19 *rpsL* vs. FA19 *penA41*

B

FA19 *rpsL* vs. FA19 *penA89*
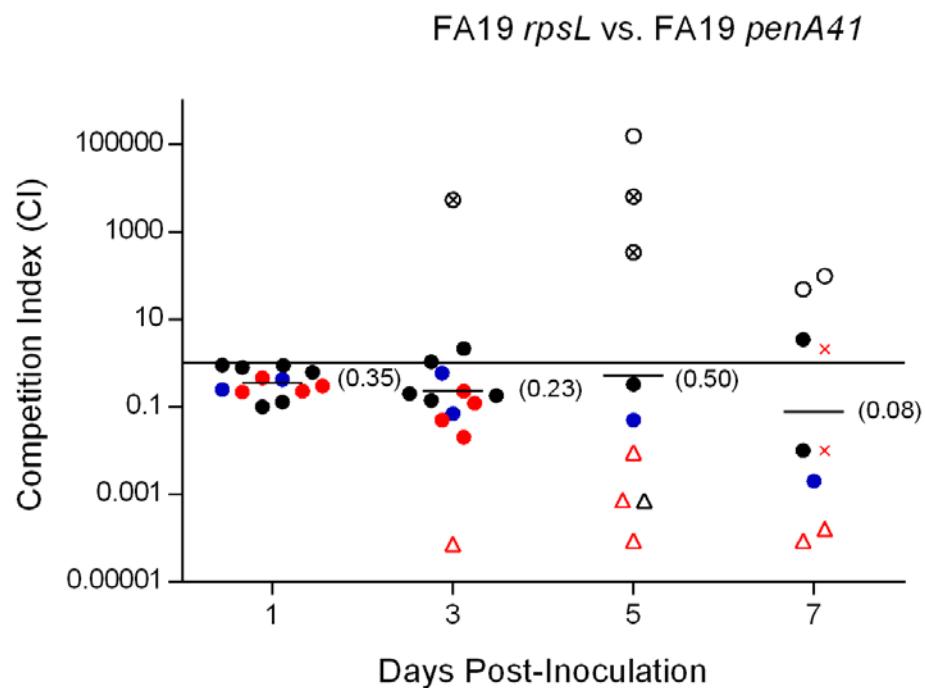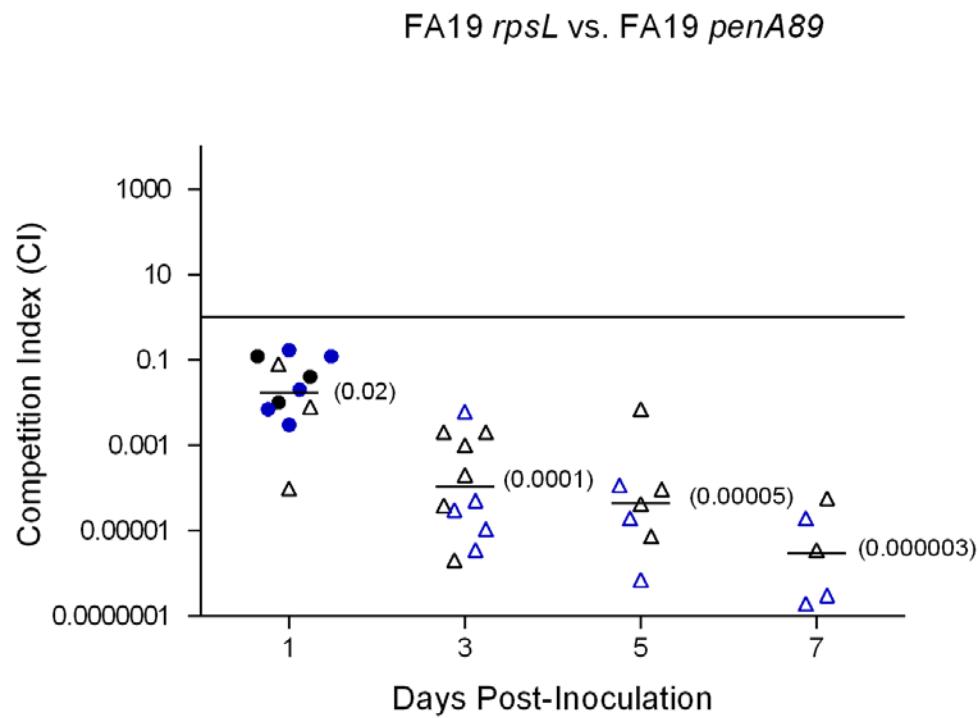
3.1c Phenotypic Characterization of Compensatory Mutant Strains Under *in vitro* Conditions

We next sought to characterize the putative compensatory mutant strains at the phenotypic level. We previously reported that mutations in the transcriptional repressor (*mtrR*) or its promoter region enhance gonococcal fitness in the mouse model and that *mtr* mutations can be selected *in vivo*. These *mtrR* mutations increase gonococcal resistance to macrolide antibiotics, such as erythromycin, (84; 170; 171). We have also found that mutations in *gyrA* that increased gonococcal resistance to the fluoroquinolones also increased fitness *in vivo* (84). Our first step therefore, was to determine the MIC of erythromycin and ciprofloxacin for each mutant as a marker for these known resistance mutations that confer increased fitness in the mouse model. No differences in the MICs of these antibiotics against the FA19 *penA41* parent strain and the four compensatory mutant strains were observed (MICs: Erythromycin 0.38 µg/mL; Ciprofloxacin 0.03 µg/mL). We conclude that *in vivo*-selected *mtrR* or *gyrA* mutations were not responsible for the observed increased *in vivo* fitness of the mutant strains (Table 4). As predicted, the MIC of Cro (0.5 µg/mL) against each of the four compensatory mutant strains was also the same as that against the parent strain FA19 *penA41* (Table 4).

To test whether the compensatory mutant strains were more fit than parent strain FA19 *penA41 in vitro*, and to assess whether these mutant strains can also outcompete a Cro[S] strain carrying the wild-type *penA* allele, we competed each compensatory mutant against strains FA19 *penA41* and FA19 *rpsL*. Strain FA19 *penA41* was marked with a Cm resistance gene (*cat*) as described in the Materials and Methods to allow us to distinguish between each compensatory mutant strain and this parent strain, since both are resistant to Cro. The *cat* gene had no effect on the *in vitro* growth of strain FA19 *penA41* (data not shown) or *in vitro* fitness in competitive co-culture (141). Mutant strains LV41A and LV41E showed a strong fitness advantage compared to

the Cro^R parent strain when co-cultured *in vitro*, with a 7-fold and 17-fold increase in the CI for these mutant strains, respectively, at 8 hours of incubation (Table 6). Mutant strains LV41A and LV41E were equally fit as the Cro^S parent strain FA19 *rpsL*. In contrast, mutant strain LV41B showed no fitness difference compared to the parent strain FA19 *penA41*, and was slightly less fit (2-fold) than Cro^S FA19 *rpsL* bacteria. Mutant strain LV41C was slightly more fit (2-fold) than parent strain FA19 *penA41*, and slightly less fit (2-fold) than FA19 *rpsL*. These results suggest that each of the mutant strains carry unique compensatory mutations, with those carried by LV41A and LV41E providing the greatest growth advantage.

We also performed noncompetitive growth curves in supplemented GC broth under aeration to examine more closely the growth characteristics of the *in vivo*-selected mutant strains. As expected, mutant strain FA19 *penA41* grew significantly slower than its Cro^S parent strain FA19 *rpsL*, as measured by the time it took to reach an OD$_{600}$ of 0.8- late logarithmic phase of growth (278 min versus 205 min, p $\leq$ 0.001, Table 5, Figure 9A). In contrast, mutant strains LV41A, LV41C, and LV41E reached an OD$_{600}$ of 0.8 significantly faster than the parent FA19 *penA41* strain (*p* values, 0.018, 0.003, and 0.0006, respectively). Notably, mutant strain LV41C exhibited a unique growth profile, characterized by rapid growth during the logarithmic phase, followed by a plateau, after which the OD$_{600}$ quickly began to plummet. Mutant strains LV41A and LV41B both reached an OD$_{600}$ of 0.8 significantly slower than Cro^S strain FA19 *rpsL* (*p* values 0.0002 and 0.002, respectively. There was no significant difference between mutant strains LV41C, LV41E, and Cro^S strain FA19 *rpsL* (*p* values 0.260 and 0.371, respectively). No difference in viable counts was observed between strains until mid-log phase (Figure 9B). At 240 minutes, the number of viable LV41C bacteria recovered plummeted, mirroring what we observed in optical density (Figure 9A). We observed the largest variation between strains

beginning at 300 minutes, at which time the slower growth of FA19 *penA41* and decreased recovery as compared to all other strains became most evident (Figure 9).

Light microscopy images using phase contrast taken at mid-log phase appeared to show clustering in compensatory mutant strains LV41A, LV41B and LV41E (Figure 10). The clustering phenotype was not observed in mutant strain LV41A by scanning electron microscopy, however (Figure 11). We also isolated the peptidoglycan sacculus of FA19 *rpsL,* FA19 *penA41,* and LV41A to determine if the observed difference in optical density might be linked to differences in the peptidoglycan layer of the different mutant strains. This possibility was supported by the demonstration by Antignac *et al.* of a correlation between the percentage of muropeptides carrying a pentapeptide side chain and increased resistance to penicillin G in strains of *Neisseria meningitidis* (10). Qualitatively, we saw no differences in the observed peaks between strains FA19 *rpsL,* FA19 *penA41,* and LV41A that would indicate differences in peptidoglycan composition (Figure 12). Based on these experiments, we conclude that peptidoglycan differences are not responsible for the increased optical density observed in mutant strain LV41A.

3.1d Characterization of Compensatory Mutant Strains *in vivo*

Results from competitive and noncompetitive broth cultures are consistent with the capacity of compensatory mutations to restore growth to a Cro[R] strain at a level that is equal to or higher than the resistant parent strain (Table 5 and Table 6). Additionally, the mutations in LV41A, LV41C and LV41E restored *in vitro* fitness to levels similar to Cro[S] FA19 *rpsL*. To examine whether these compensatory mutant strains could compete with the Cro[R] and Cro[S] parent strains *in vivo,* we performed competitive murine infections. Each of the compensatory mutant strains showed a dramatic fitness advantage *in vivo* over the Cro[R] parent strain FA19

penA41, with CIs reflecting a 300- to 800-fold increase in fitness (Figure 13 and Table 6). Some

of the Cro^R compensatory mutant strains also had a fitness advantage over Cro^S strain, albeit not

as dramatic, with 2- to 4-fold increases in fitness detected by day seven of infection for LV41A,

LV41C and LV41E compared to FA19 *rpsL*. We did not observe this fitness benefit in LV41B

(Figure 14 and Table 6).

Figure 9. Growth curves for FA19 *penA41* carrying different compensatory mutations.

Strains were grown over an 8-hour period at 37°C with agitation. Aliquots were taken each hour (A) for analysis, or every two hours (B) for quantitative culture. Results shown are a mean combination of six independent experiments. The error bars show the SEM.

A
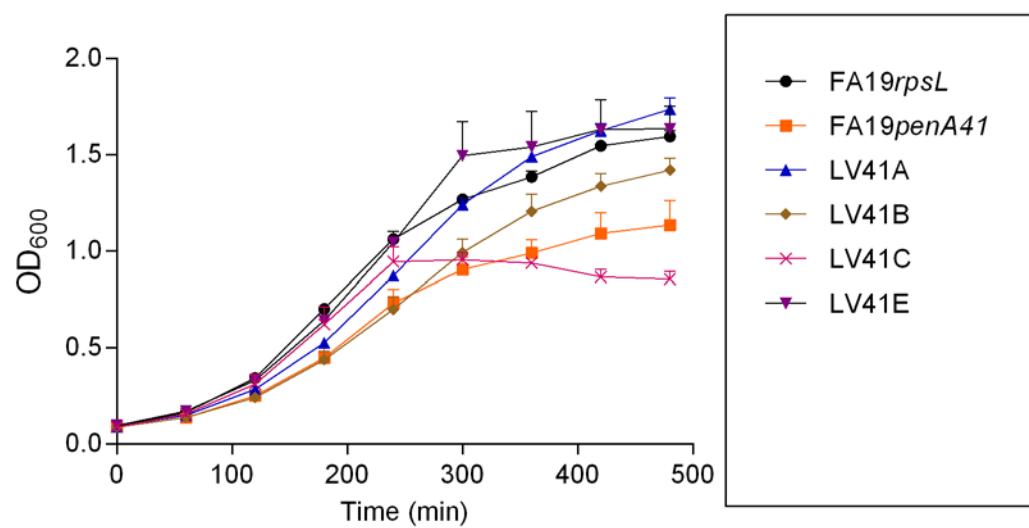
B
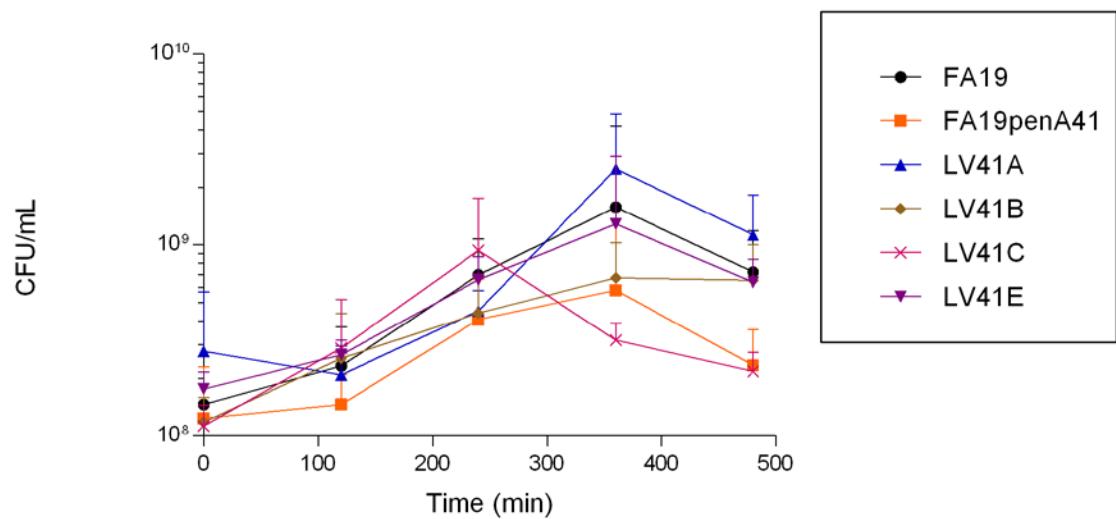
Table 5. Growth kinetics of compensatory mutant strains compared to Cro^S and Cro^R parent strains

Bacterial Strain	Time to Reach OD$_{600}$ = 0.8		
	Minutes ± SE	p value[a]	
		vs. FA19 *rpsL*	vs. FA19 *penA41*
FA19 *rpsL*	205.2 ± 3.7	--	0.001[c]
FA19 *penA41*	278.3 ± 15.6	0.001[b]	--
LV41A	233.3 ± 3.3	0.0002[b]	0.018[c]
LV41B	262.5 ± 13.5	0.002[b]	0.461
LV41C	214.0 ± 6.4	0.260	0.003[c]
LV41E	200.0 ± 3.2	0.317	0.0006[c]

Overall significance was determined by a one-way analysis of variance, followed by a student's *t*-test to determine significance between individual strains.
[a]*P* value compares each strain verses FA19 *rpsL* or FA19 *penA41,* student's *t*-test.
[b]Growth is significantly slower than Cro^S strain FA19 *rpsL*.
[c]Growth is significantly faster than Cro^R strain FA19 *penA41*

Table 6. *In vivo* and *in vitro* CIs for compensatory mutant strains versus Cro[S] or Cro[R] parent strains

Bacterial Strain	Fitness Relative to FA19 *rpsL*		Fitness Relative to FA19 *penA41, cat*	
	In vitro CI	*In vivo* CI	*In vitro* CI	*In vivo* CI
FA19 *penA41*	0.07	0.08	N/A[a]	1.0
LV41A	1.0	3.6	6.8	871.0
LV41B	0.2	0.4	1.8	480.0
LV41C	0.6	2.3	1.8	392.0
LV41E	1.0	2.0	16.4	277.0

[a]Respective strain mixture was not tested competitively; marked as not applicable (N/A)

Figure 10. Phase contrast microscopy of select compensatory mutant strains.

       Images of phase contrast light microscopy taken at mid-log phase of growth are shown for (A) FA19 *rpsL*, (B) FA19 *penA41* and (C-F) compensatory mutant strains. White arrows indicate cell clustering.
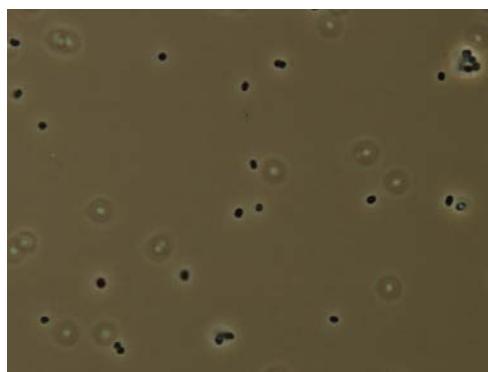
A   FA19   B   FA19 *penA41*
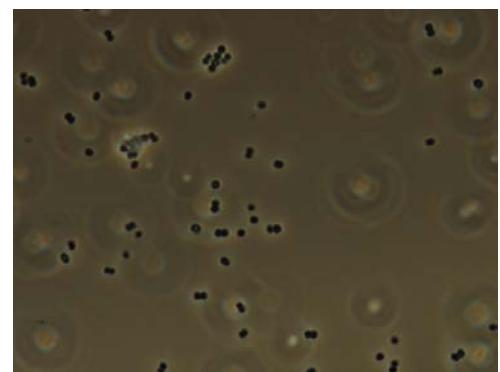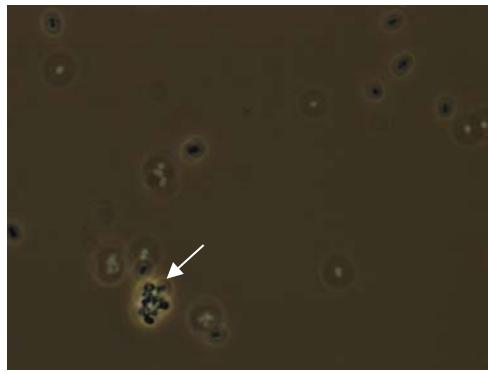
C   LV41A   D   LV41B
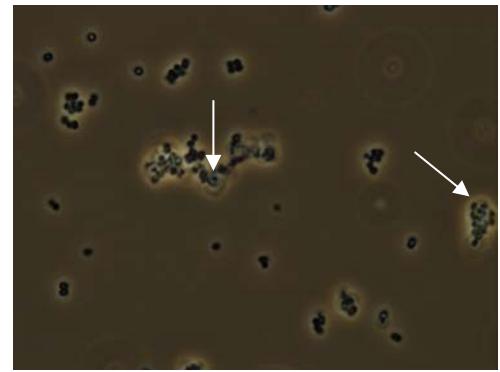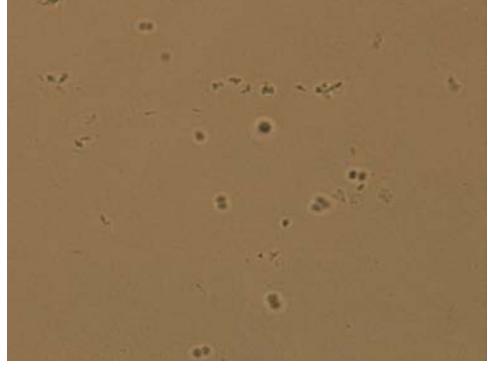
E   LV41C   F   LV41E
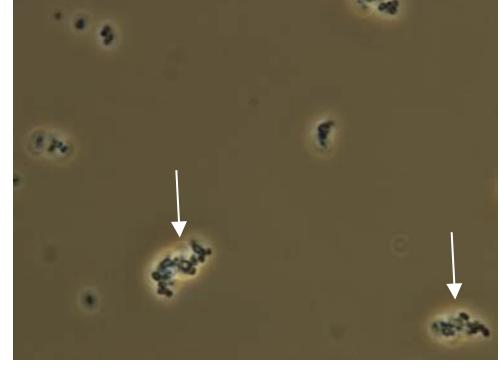
Figure 11. Scanning electron microscopy of select compensatory mutant strains.

Scanning electron micrographs taken at mid-log phase of growth are shown for (A) FA19 *rpsL*, (B) FA19 *penA41*, and (C) LV41A. Red arrows indicate normal cell division. Images are shown at both low (3.00 K X) and high (20.00 K X) magnification. Scanning electron microscopy was performed in collaboration with R. Nicholas and G. DuChamp, University of North Carolina, Chapel Hill (unpublished data).
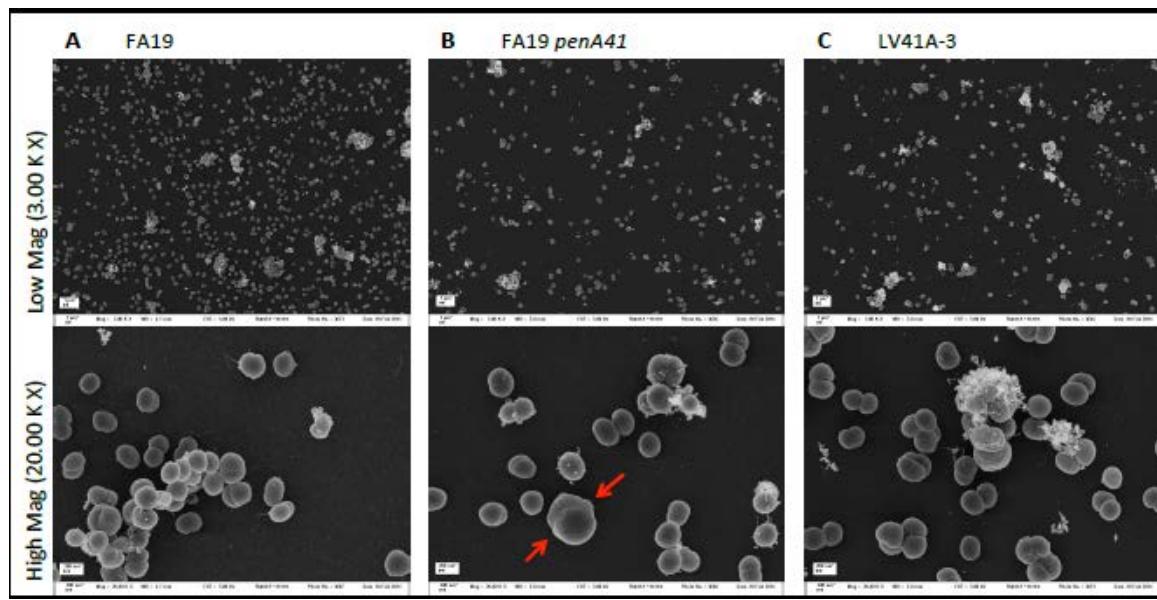
Figure 12. Peptidoglycan analysis of select compensatory mutant strains.

Reverse-phase HPLC analysis used a linear gradient from 0-15% of HPLC-grade water with 0.1% trifluoroacetic acid to acetonitrile applied for 70 minutes to a column (4.6 x 250 nm; 5 µm particles) at room temperature with a 0.6 mL/min flow rate. UV peaks were detected at 210 nm. A 50 µL injection volume of peptidoglycan sample was used. Reverse-phase HPLC was performed in collaboration with R. Nicholas and G. DuChamp, University of North Carolina, Chapel Hill (unpublished data).
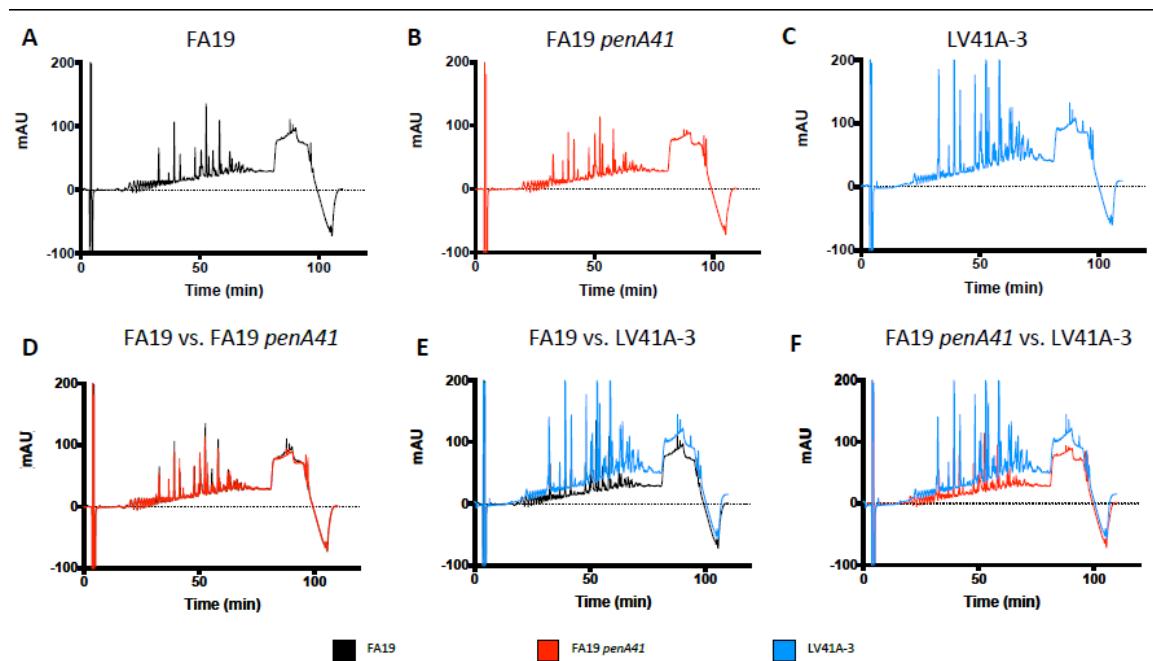
Figure 13. *In vivo* competition of strains harboring compensatory mutation(s) against the Cro^R parent strain carrying a *cat* marker.

Results of competitive murine infection experiments with Cro^R strain FA19 *penA41, cat* versus compensatory mutant strains (A) LV41A, (B) LV41B, (C) LV41C, or (D) LV41E. The mice were infected with similar numbers of each strain and the competition index was determined over a 7-day period. Each shape indicates an individual mouse. Bars indicate geometric mean (also indicated by numbers in parentheses). Open circles indicate that only mutant bacteria were recovered. The results are a combination of two independent experiments, which are indicated by different colors.

A

FA19 *penA41, cat* vs. LV41A

B

FA19 *penA41, cat* vs. LV41B

C

FA19 *penA41, cat* vs. LV41C

D

FA19 *penA41, cat* vs. LV41E
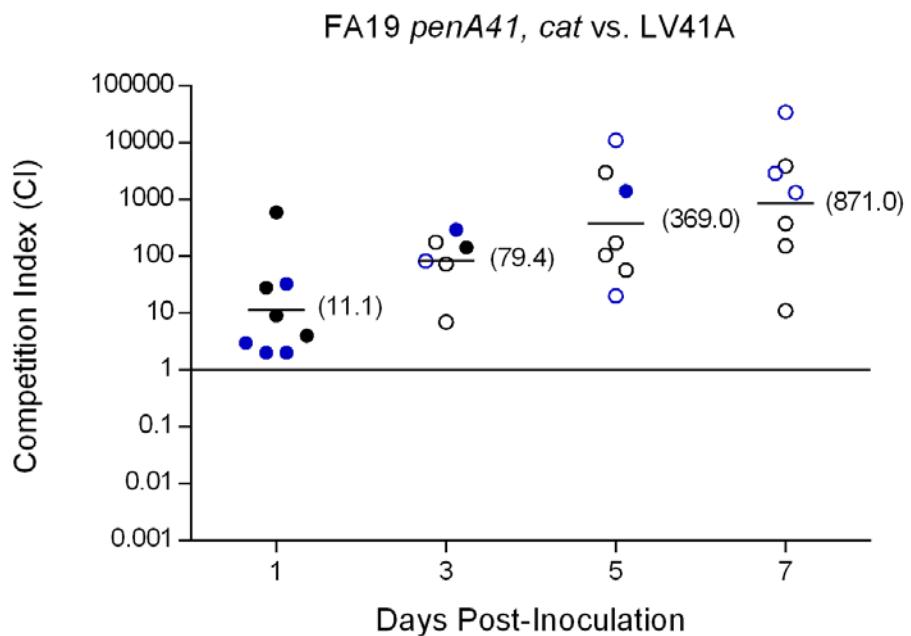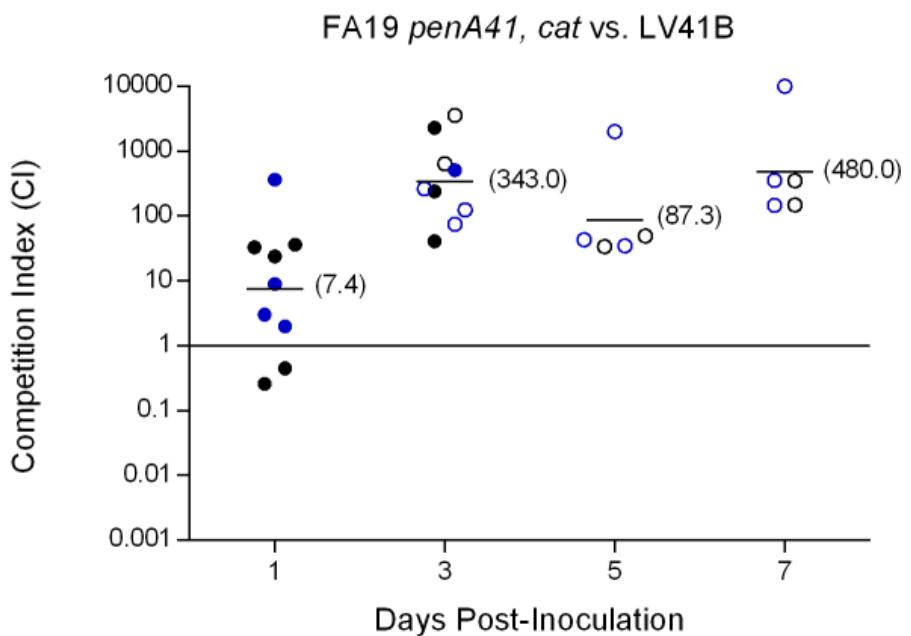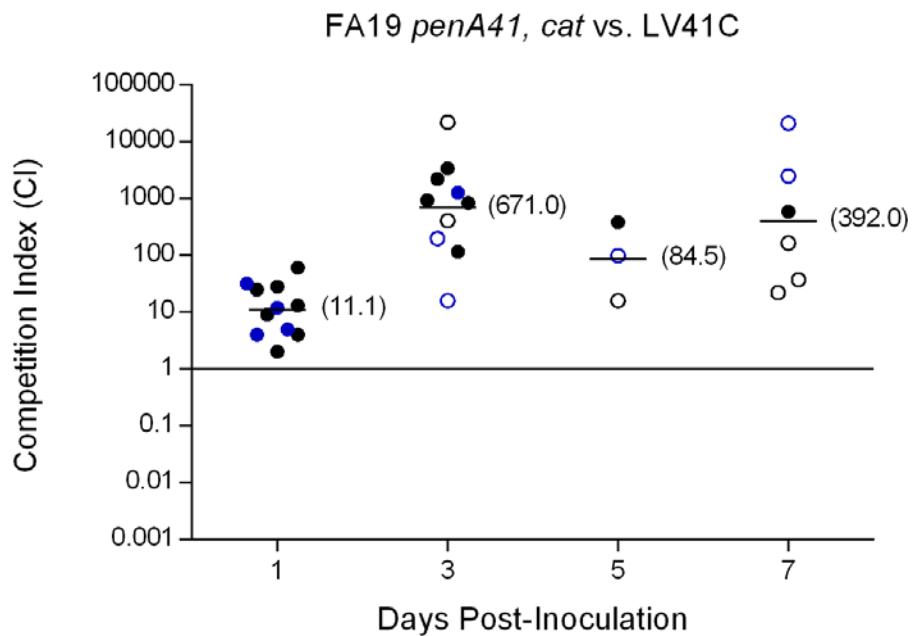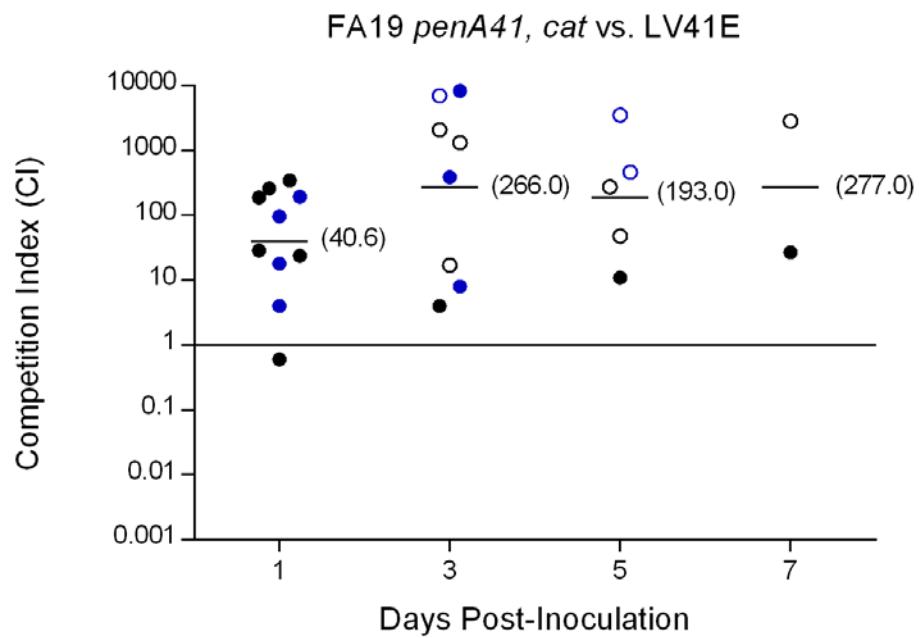
Figure 14. *In vivo* competition of strains harboring compensatory mutation(s) against Cro[S] strain FA19 *rpsL*.

Results of competitive murine infection experiments with Cro[S] strain FA19 *rpsL* versus compensatory mutant strains (A) LV41A, (B) LV41B, (C) LV41C, or (D) LV41E. Mice were infected with similar numbers of each strain and the competition index was determined over a 7-day period. Each shape indicates an individual mouse. Bars indicate geometric mean. Open circles indicate that only mutant bacteria were recovered. Open triangles indicate only FA19 *rpsL* were recovered.

A

FA19 *rpsL* vs. LV41A

B

FA19 *rpsL* vs. LV41B

C

FA19 *rpsL* vs. LV41C

D

FA19 *rpsL* vs. LV41E
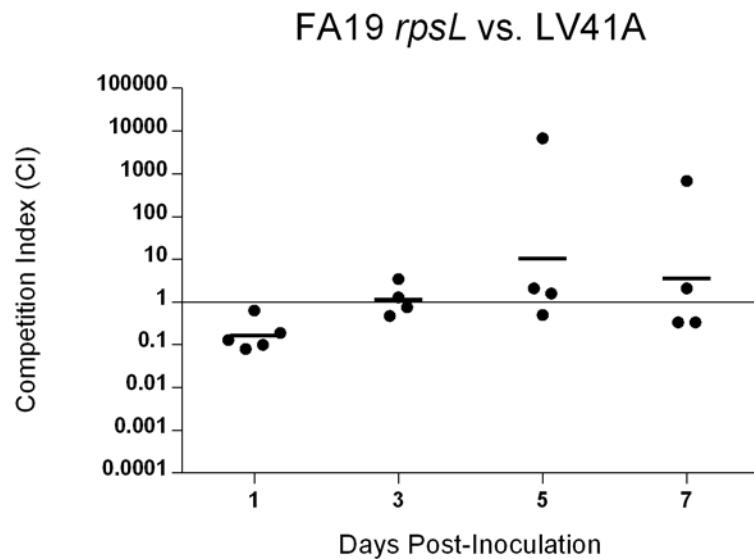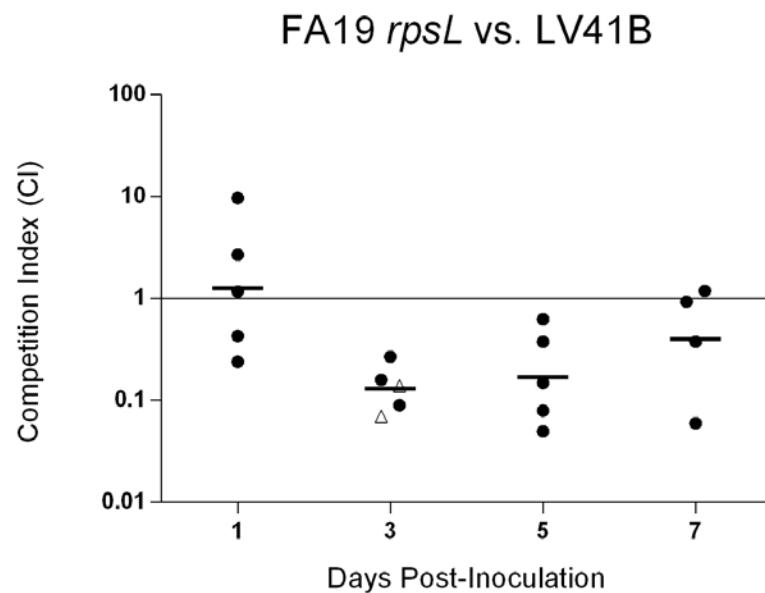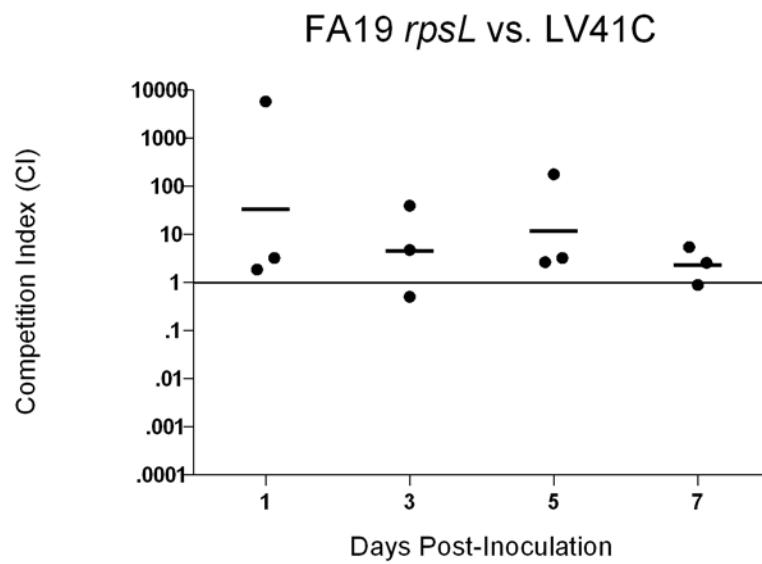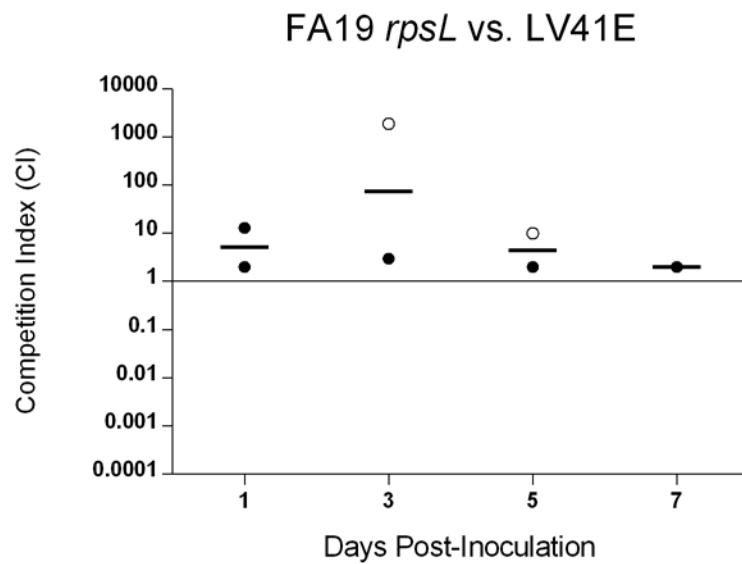
3.1e Identification of Mutations in Genes Involved in Metabolism or Small Molecule Transport

To identify the mutation(s) that might be responsible for the observed compensations to strain FA19 *penA41*, we obtained the whole genome sequences of strains FA19 *rpsL*, FA19 *penA41*, and the four compensatory mutant strains. Strain FA19, the reference strain, was re-sequenced and annotated using the Pac-Bio system. We mapped single-nucleotide variations (SNVs), and the alignment and analysis were repeated using CLC Genomics Workbench to verify reproducibility of results. As expected, the *rpsL* gene was identified in strain FA19 *rpsL* and all of the Cro^R mutant strains but not in the reference FA19 strain (Table 7). Differences in pilin alleles were also detected; this result was not unanticipated based on the antigenic variability of the *pilE* gene. We identified three other mutations in mutant LV41A, one of which was a deletion mutation in *mleN,* which encodes a malic/Na+- lactate antiporter and two SNVs in intergenic regions of the genome. We found these same three mutations in mutant LV41B. One of the SNVs, the T→G transversion, was also found in mutant LV41C. Mutant LV41C had two additional mutations: a G→A transition located in the *acnB* gene, which encodes the bifunctional aconitate hydratase 2/2 methylisocitrate dehydratase, and a frameshift mutation in PROKKA_02121, which may encode a hypothetical protein related to the glycosyl transferase-1 family of enzymes. LV41E had a single C→T transition located in an intergenic region.

Table 7. Nucleotide changes identified in compensatory mutant strain derivatives of FA19 *penA41*

Bacterial Strain	Genetic Position	Common Name	Mutation	Annotation
LV41A	2115410	*mleN*	*mleN* $_{\Delta A467}$[a]	Malic/Na+-lactate antiporter
	1849145	--	SNV[b] T$\rightarrow$G	Intergenic Region
	1849152	--	SNV G$\rightarrow$A	Intergenic Region
LV41B	2115410	*mleN*	*mleN* $_{\Delta A467}$	Malic/Na+-lactate antiporter
	1849145	--	SNV T$\rightarrow$G	Intergenic Region
	1849152	--	SNV G$\rightarrow$A	Intergenic Region
LV41C	9664470	*acnB*	*acnB*$_{G348D}$	Aconitate hydratase 2/2 methylisocitrate dehydratase
	2065357	PROKKA_02121	PROKKA_02121$_{R165fs}$[c]	Hypothetical protein related to glycosyl transferase-1
	1849145	--	SNV T$\rightarrow$G	Intergenic Region
LV41E	1849201	--	SNV C$\rightarrow$T	Intergenic Region

[a]Δ refers to codon deletion
[b]Single nucleotide variation (SNV)
[c]Frameshift mutation (fs)

3.1f Introduction of the *acnB*$_{G348D}$ Mutation into FA19 *penA41* and FA19 *penA89* Confers Increased Fitness Relative to the Resistant Parent Strain

To determine whether the mutated *acnB* allele was responsible for the observed fitness differences *in vivo*, we moved the G$_{348}$D mutation into the Cro^R parent strain FA19 *penA41* and the Cro^S strain FA19 *rpsL*. The construct carrying the G$_{348}$D mutation also contained a kanamycin cassette, which provided a selective marker (Figure 5). Strain FA19 *penA41, acnB*$_{G348D}$ was 6-fold more fit than parent strain FA19 *penA41* when competed *in vivo* (Figure 15A). This result was in contrast to the original mutant LV41C, which was 400-fold more fit than parent strain FA19 *penA41, cat* by day seven of infection (Figure 13C). When competed against strain FA19 *rpsL,* the fitness of strain FA19 *penA41, acnB*$_{G348D}$ was not increased, and was 5-fold less fit than FA19 *rpsL* (Figure 15B). This is in contrast to strain LV41C, which was 3-fold more fit than strain FA19 *rpsL* by day seven of infection (Figure 14C).

We also moved the G$_{348}$D mutation into strain FA19 *penA89*, which was unable to survive past day three of infection in earlier competition experiments with the Cro^S parent strain (Figure 6B). Mutant FA19 *penA89, acnB*$_{G348D}$ out-competed FA19 *penA89* with a 50-fold increase in fitness detected (Figure 15C and Table 6). We also competed FA19 *penA89, acnB*$_{G348D}$ against strain FA19 *rpsL*, and fitness of the mutant was 100-fold less fit than FA19 *rpsL* (Figure 15D).

In conclusion, when reintroduced into mutant FA19 *penA41* the *acnB*$_{G348D}$ mutation first isolated from strain LV41C increased fitness *in vivo* relative to that of parent strain FA19 *penA41*, although levels were not as high as exhibited by LV41C. A difference in the fitness advantage of FA19 *penA41*, *acnB*$_{G348D}$ and LV41C relative to FA19 *rpsL* was also observed. From these experiments, we conclude that other

mutations, perhaps the hypothetical protein PROKKA_02121$_{R165fs}$, may also confer increased fitness to strain FA19 *penA41 in vivo*. Interestingly, however, the *acnB*$_{G348D}$ mutation was able to rescue the *in vivo* fitness of strain FA19 *penA89* although the mutation did not restore fitness to the level of the Cro^S strain FA19 *rpsL*.

Figure 15. *In vivo* competition of FA19 *penA41* and FA19 *penA89* mutant strains harboring the *acnB*$_{G348D}$ mutation against parent strains.

Results of competitive murine infections for (A) FA19 *penA41* against FA19 *penA41*, *acnB*$_{G348D}$, (B) FA19 *rpsL* against FA19 *penA41, acnB*$_{G348D}$, (C) FA19 *penA89* against FA19 *penA89*, *acnB*$_{G348D}$, or (D) FA19 *rpsL* against FA19 *penA89, acnB*$_{G348D}$. Open circles indicate that only mutant bacteria were recovered. Open triangles indicate that only wild-type bacteria were recovered. The results are a combination of (A) two independent experiments (A) or one experiment (B, C, D), as indicated by different colors. The bar indicates the geometric mean, which is also indicated by value in parentheses.

A

FA19 *penA41* vs. FA19 *penA41, acnB*G348D

B

FA19 *rpsL* vs. FA19*penA41, acnB*G348D

C

FA19 *penA89* vs. FA19 *penA89, acnB*$_{G348D}$

D
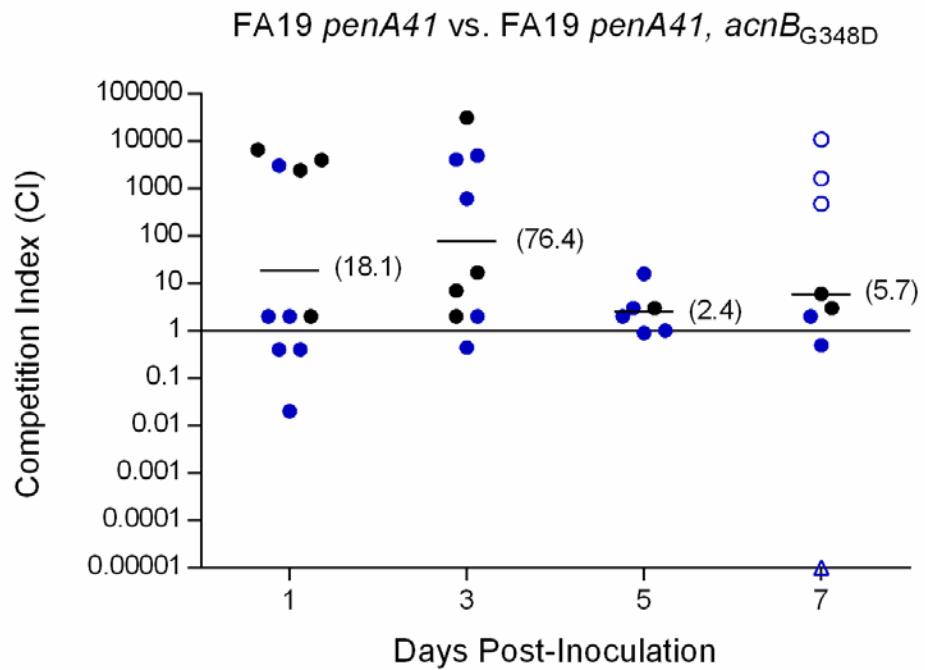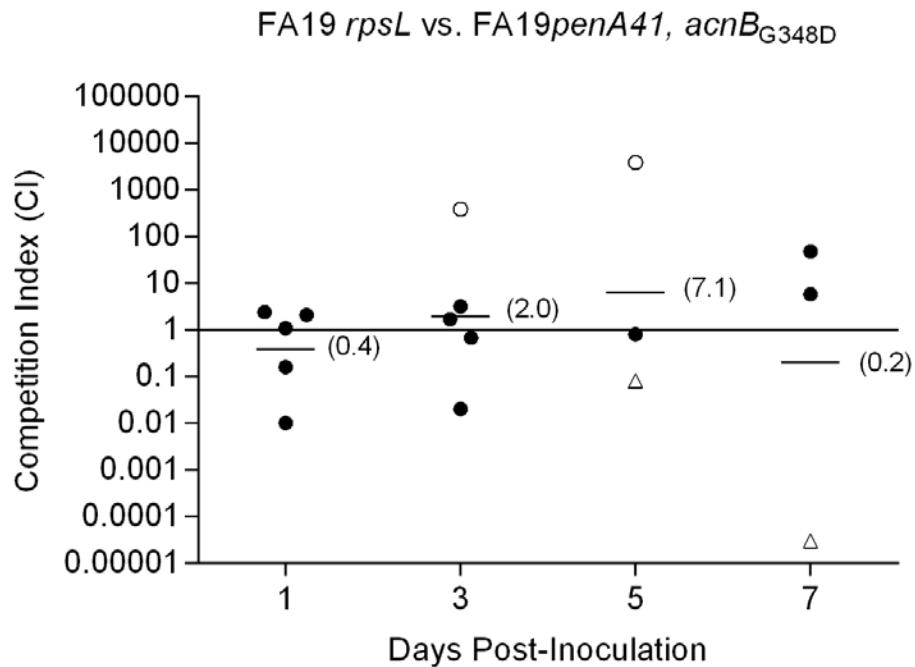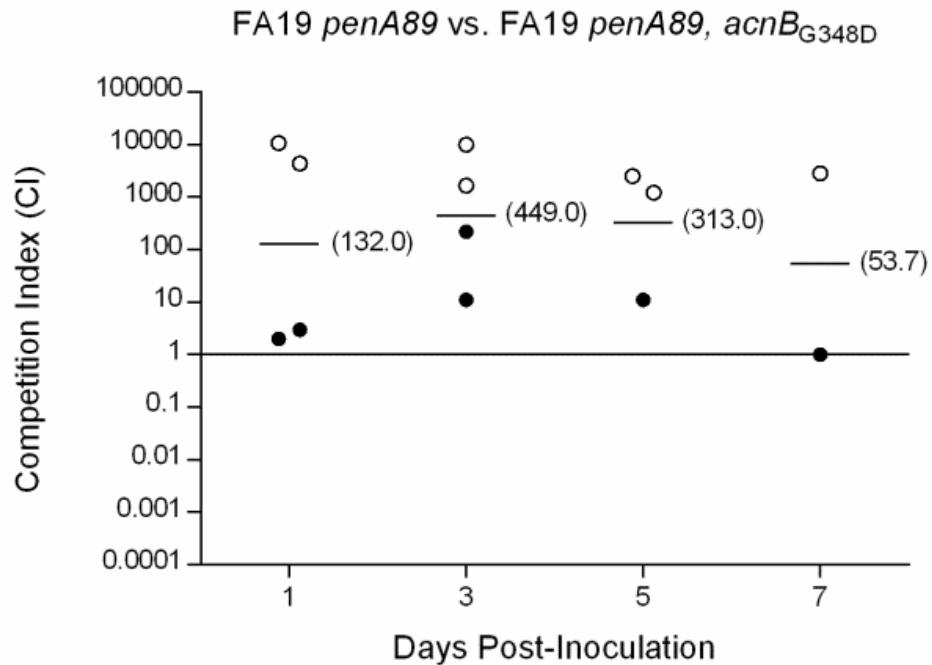
FA19 *rpsL* vs. FA19 *penA89, acnB*$_{G348D}$
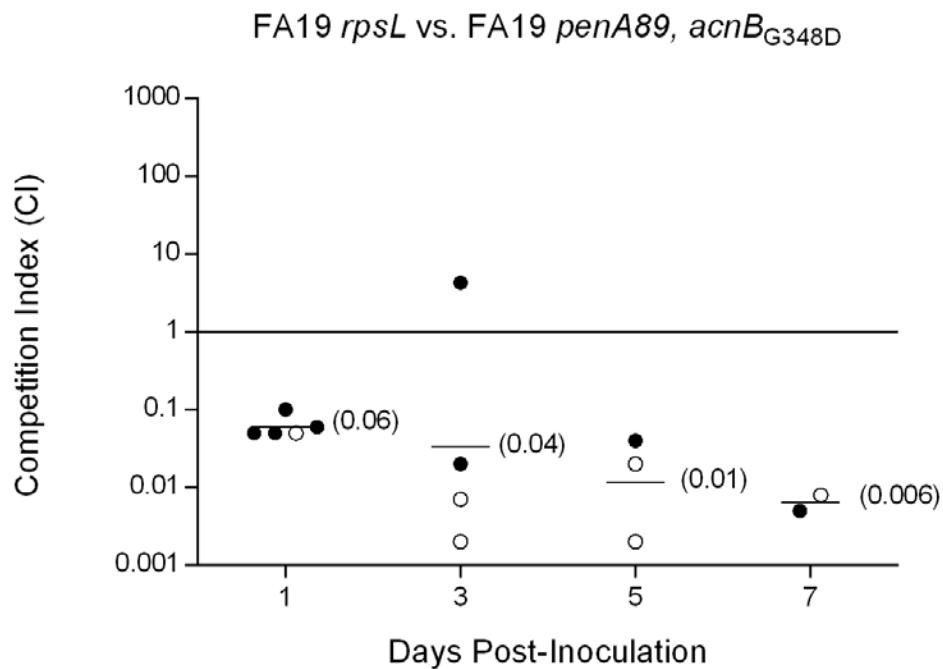
3.1g Carbohydrate Utilization and Growth in Iron-limited Conditions of the *acnB*$_{G348D}$ Mutant Strain

AcnB is critical for the tricarboxylic acid cycle (TCA) as well as 2-methylcitrate cycle activity. The G→A substitution is located in a glycine at position 348 in the wild-type protein that is highly conserved across Gram-negative bacteria, and is not located near the active site of the enzyme (personal communication, R. Nicholas). To study the function of AcnB$_{G348D}$, AcnB was purified from wild-type and LV41C bacteria, and its ability to catalyze the conversion of citrate to isocitrate, and the reverse reaction of isocitrate to citrate was tested. Michaelis-Menten kinetics showed that the G$_{348}$D mutant is bi-directionally impaired, in both the catalysis of citrate to *cis*-aconitate, and the opposite reaction of isocitrate to *cis*-aconitate (personal communication, R. Nicholas). *N. gonorrhoeae* utilizes glucose during log-phase growth through the Embden-Meyerhoff pathway of metabolism, not the TCA cycle (62; 127). The TCA cycle is not active until glucose has been depleted and the bacteria are growing on acetate (62; 127). We therefore hypothesized that the rapid exponential growth of mutants carrying the *acnB*$_{G348D}$ allele (LV41C and FA19 *penA41, acnB*$_{G348D}$), followed by a quick transition to the stationary phase may be due to rapid depletion of glucose by the mutants. We therefore measured glucose consumption using an assay that measures the amount of NADPH formed when D-glucose is metabolized to D-gluconate-6-phophate. The amount of NADPH formed is stoichiometric to the amount of D-glucose present. We found no difference in glucose consumption between strains, from which we conclude that the mutation in AcnB does not affect glucose consumption (Figure 16).

The above results do not explain the *in vitro* growth kinetics of strains carrying the *acnB*$_{G348D}$ mutation. It is also not clear why the fitness advantage appears more pronounced *in vivo* than *in vitro* (Table 6). It is possible that conditions *in vivo* may enhance the fitness advantage exhibited by gonococci carrying the *acnB*$_{G348D}$ allele. One such condition may be iron limitation, which is known to occur during experimental murine infection (72) and during cervical infection in women (98). Recently it was reported that *Helicobacter pylori* aconitase acts as a pleiotropic post-transcriptional regulator during iron-stressed conditions (12). Therefore, we hypothesized that the stronger fitness benefit conferred by the *acnB* mutation *in vivo* compared to *in vitro* may be due to a regulatory function that is induced by low iron. We tested this hypothesis by growing Gc under iron-limited conditions *in vitro* in independent growth and competitive co-culture (Figure 17). No difference in growth was observed in independent culture (Figure 17A). We also observed no differences in fitness between strain LV41C and either FA19 *rpsL* (Figure 17B) or FA19 *penA41, cat* (Figure 17C) when co-cultured under iron-limited conditions.

In order to understand better the *in vivo* fitness advantage conferred by the *acnB*$_{G348D}$ mutation, we tested two metabolic conditions that may affect *in vivo* growth: glucose consumption and iron-limitation. We determined that there was no difference in glucose consumption between strains, nor was there a significant difference amongst strains during iron-limited growth *in vitro*.

107

Figure 16. Glucose consumption of FA19 *rpsL,* FA19 *penA41,* and strains harboring the $acnB_{G348D}$ mutation.

Strains were grown over an 8-hour period at 37°C with agitation at 220 RPM in supplemented Gc broth, as described previously. The glucose assay was performed as described in the Materials and Methods. Growth over 8-hours (A). The ratio of the OD_{340} to OD_{360}, which uses the production of NADPH as a surrogate for glucose consumption (B).

A

B
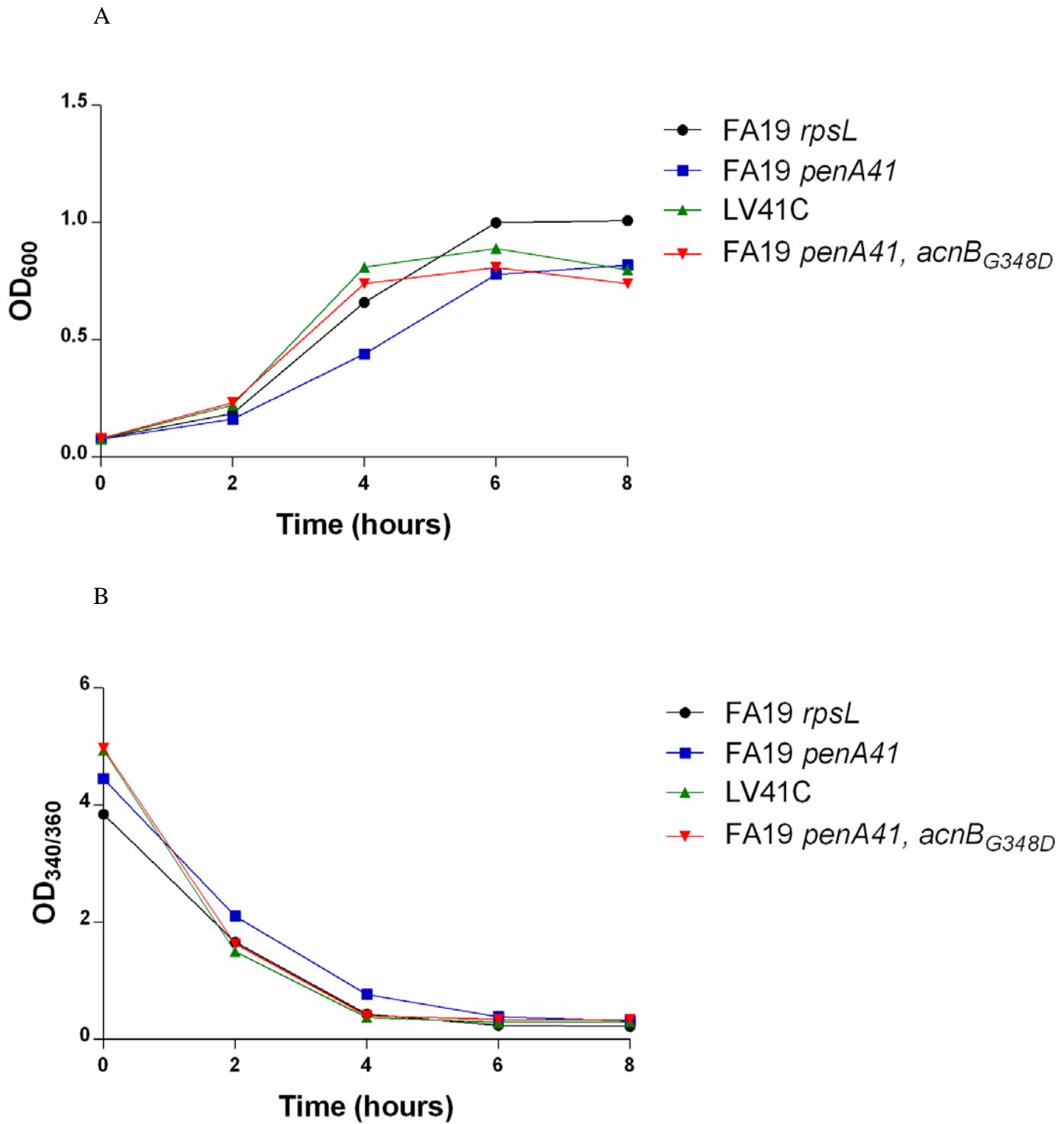
Figure 17. Growth and competition during iron-deplete conditions.

       Bacteria were grown under iron-replete conditions at 37°C with shaking at 220 RPM until mid-log phase. Cultures were then inoculated into fresh, iron-deplete media containing 50 μM DFM (A). Bacteria were grown under iron-replete conditions at 37°C with shaking at 220 RPM until mid-log phase. Cultures were then inoculated into fresh, iron-deplete media containing 50 μM DFM or GC media with Kellogg's supplements I and II. Competitive co-culture of Cro^S FA19 *rpsL* and compensatory mutant LV41C (B), and competitive co-culture of Cro^R FA19 *penA41, cat* and compensatory mutant LV41C (C) are shown.

A

B

FA19 *rpsL* vs. LV41C

C
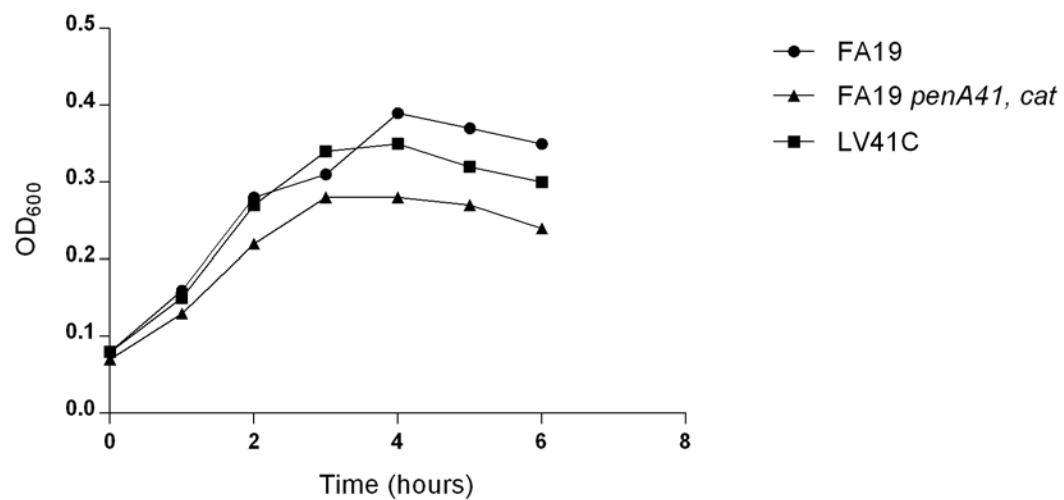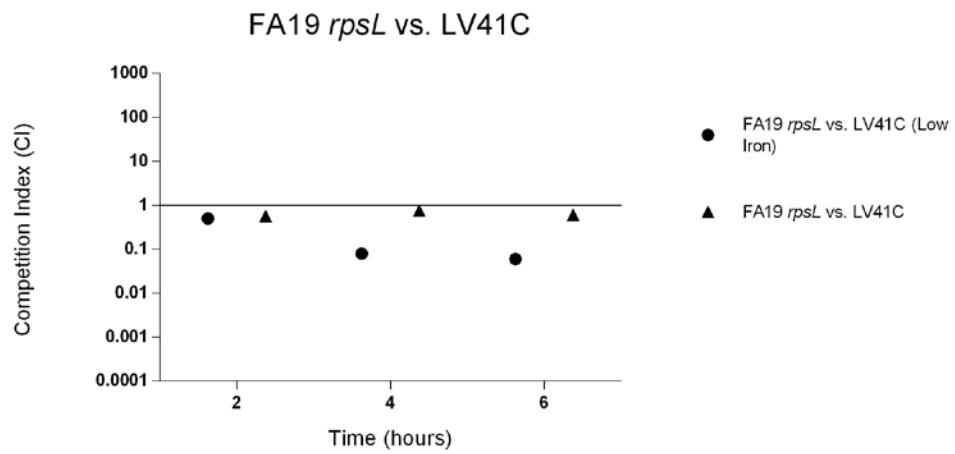
FA19 *penA41, cat* vs. LV41C
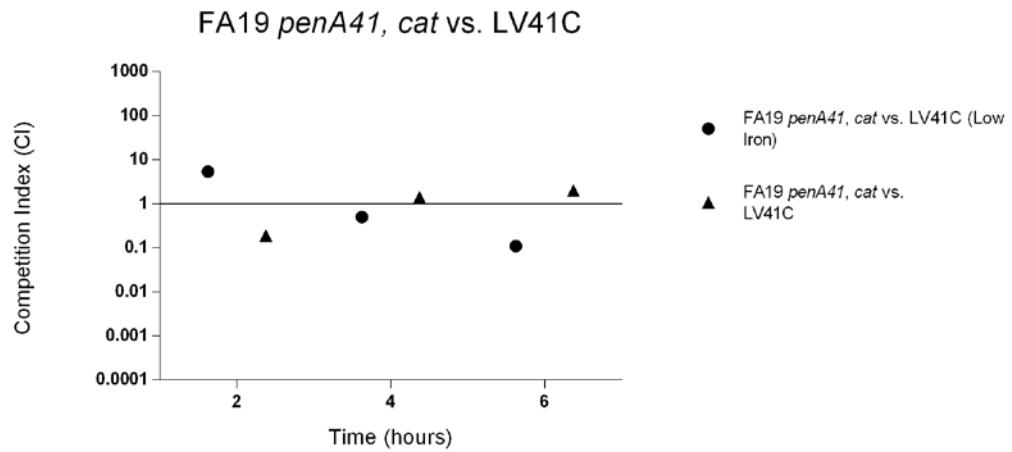
3.2 TRANSFER OF ANTIBIOTIC RESISTANCE

3.2a Transformation Efficacy of Antibiotic Resistance Alleles *in vitro* Using Purified Genomic DNA (gDNA)

Mixed infections may lead to the spread of antibiotic resistance, but this has not been demonstrated with pathogenic species of *Neisseria* in an *in vivo* model. We hypothesized that during mixed infection, resistance determinants known to increase bacterial fitness *in vivo* would be successfully transferred more frequently *in vivo* to susceptible strains than would resistance determinants that decrease fitness.

We analyzed three resistance mechanisms to examine further the spread of resistance: Ceftriaxone (Cro) resistance conferred by the *penA41* allele, Ciprofloxacin (Cip) resistance conferred by the $gyrA_{91/95}$ mutation, and Erythromycin (Erm) resistance conferred by the mutant $mtrR_{-79}$ allele. The first step in investigating genetic transfer *in vivo* was to standardize conditions for demonstrating the transformation of these markers *in vitro.* The bacterial strains used in these experiments are described in Table 3. Gc strain OP100 was the recipient strain in all experiments and is a derivative of strain FA1090 that is marked with a Cm^R gene (Table 3) to distinguish it from the donor strains. Donor strains AK1, FA19 *penA41*, and KH15 are resistant to Cip, Cro and Erm, respectively. MIC testing was performed to identify the antibiotic concentrations needed to inhibit the recipient strain (Cip, Erm or Cro) and donor strains (Cm) (Table 8). Based on these data, we chose 0.5 µg/mL of Cm combined with 0.125 µg/mL of Cip to select OP100 transformants carrying the $gyrA_{91/95}$ allele, 0.125 µg/mL of Cro to select OP100 transformants carrying *penA41*, and 1.0 µg/mL of Erm to select transformants carrying the $mtrR_{-79}$ allele.

We first compared the *in vitro* efficacy of transformation for each resistance allele by incubating the recipient strain with increasing concentrations of gDNA from each of the resistant donor strains essentially as described (134; 136; 145). The recipient strain OP100 was incubated with 10 µg, 5 µg, 1 µg, or 500 ng of DNA to a final concentration of 2.5 µg/mL, 1.25 µg/mL, 250ng/mL, 125 ng/mL, and 50 ng/mL, respectively, and the suspension was plated on agar using the appropriate selection described above. In parallel, DNase I was added to the sample DNA and incubated for 15 minutes at room temperature, after which it was heat-deactivated, and the DNA/DNase I suspension was incubated with recipient strain OP100 as described above. We determined that 1.25-2.5 µg/mL of DNA were optimal for transformation of the *penA41* and *gyrA$_{91/95}$* alleles (Figure 18), and that the *penA41* allele was transformed more efficiently than the *gyrA$_{91/95}$* allele. As little as 50 ng/mL of gDNA could be used to transform efficiently the *penA41* and *gyrA$_{91/95}$* alleles into strain OP100. In contrast, 2.5 µg/mL of KH15 gDNA were required for integration of the *mtrR$_{-79}$* mutation, showing that it was either less efficiently taken up from the gDNA preparation or less efficiently integrated. Differences in transformation efficacy were also reflected by differences in incubation time; no Erm[R] transformants were recovered from any gDNA concentration when the bacteria and gDNA suspension was incubated for 4 hours (data not shown). In contrast, Cro[R] and Cip[R] transformants were recovered after both 4 and 6 hours of incubation. We also determined that DNase I could block the uptake of DNA at all concentrations tested as expected with natural transformation.

Table 8. Minimum inhibitory concentration of antibiotics used in transformation experiments

Strain	Antibiotic (µg/ml)			
	Chloramphenicol	Ciprofloxacin	Ceftriaxone	Erythromycin
OP100	2	<0.03	<0.03	0.25
AK1, *recA-*	0.5	0.5	<0.03	0.25
KH15, *recA-*	0.5	<0.03	<0.03	>2
FA19 *penA41, recA-*	0.5	<0.03	0.5	0.25

Figure 18. Transformation efficiency of antibiotic resistance alleles using gDNA.

      OP100 was grown over a 6-hour time course in supplemented GC broth to mid-log phase, and then 1 mL of culture was inoculated into 3 mL of fresh broth. Samples were treated concurrently with either gDNA from one of the respective donor strains, or gDNA that had been treated with DNase I. Aliquots were plated after six hrs incubation onto media containing Cm plus Cip ($gyrA_{91/95}$), Erm ($mtrR_{-79}$) or Cro ($penA41$) to select the desired transformants.

Genomic DNA Concentration
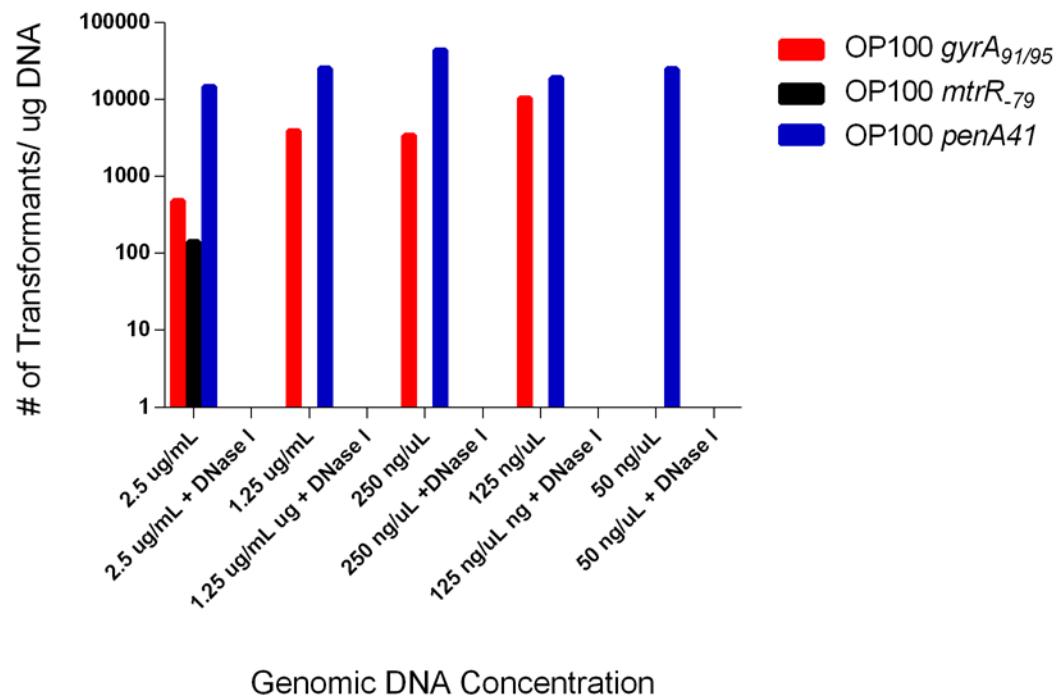
3.2b Transformation Efficacy of Antibiotic Resistance alleles *in vitro* Using Whole Bacteria

We next measured the transformation efficacy of the three resistance alleles when the recipient and donor strains were co-cultured in broth. To ensure that DNA transfer occurred uni-directionally, that is only from donor strain to recipient strain, we constructed donor strains that were recombination deficient by introducing an insertionally inactivated gonococcal *recA* gene (*recA*::*aphA-3*) and selecting for kanamycin resistance as described in the Materials and Methods (102; 143; 144).

The *recA-* strains were verified by PCR; strains that contained the wild-type *recA* gene were identified by amplification of a ~1 Kb fragment, and those in which the *recA::aphA3* gene replaced the wild-type allele yielded a ~2 Kb fragment (Figure 19).

Figure 19. Confirmation of *recA-* donor strains by PCR amplification.

PCR products from Cm^R transformants of strains FA19 *penA41*, KH15 and AK1 and their respective parent strains were separated on a 0.8% agarose gel. The amplicons from the transformants were ~ 1 kB larger than the wild type *recA* gene as predicted from the expected size of the *recA::aphA-3* gene.
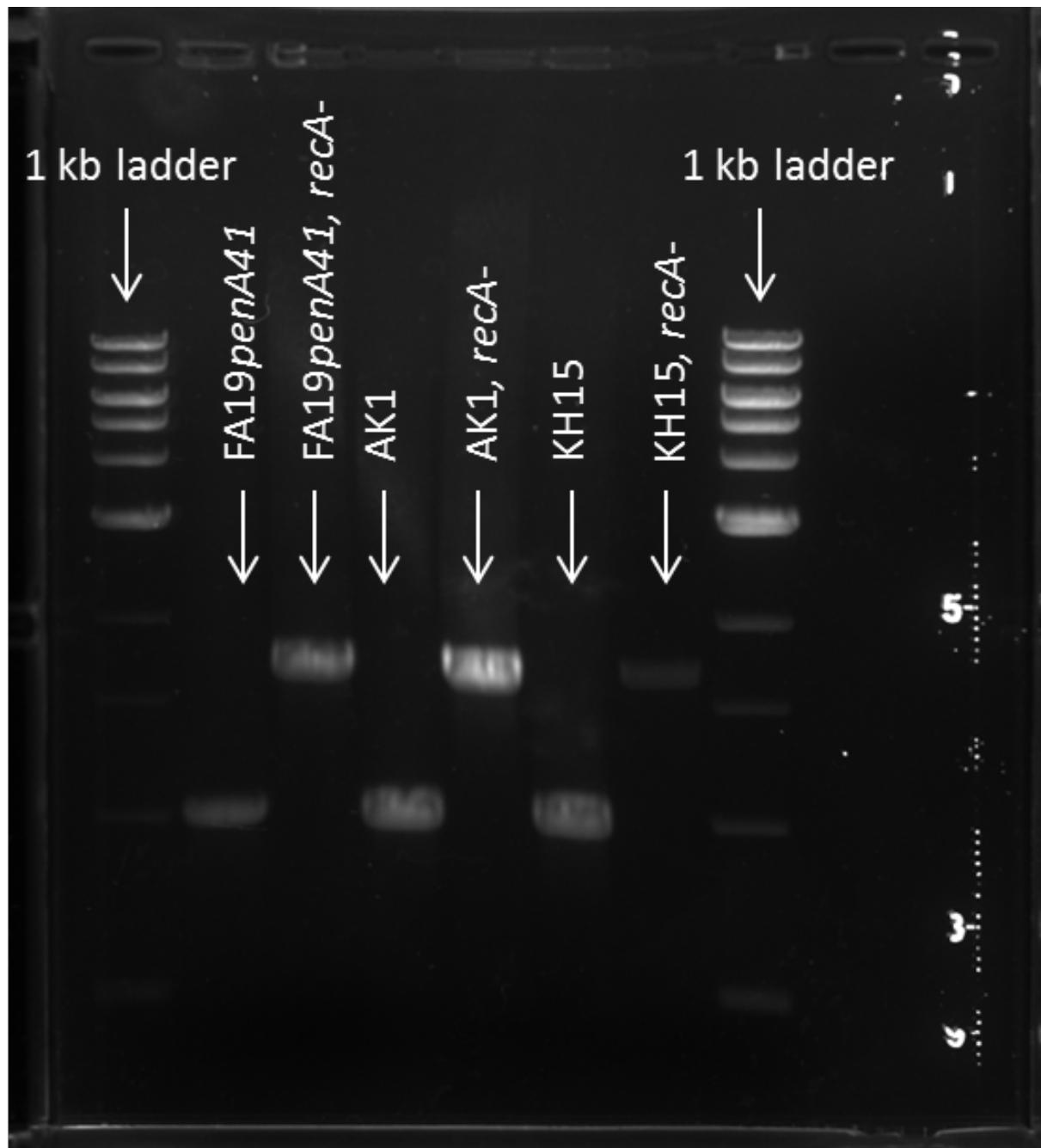
We then co-cultured recipient and recombination-deficient donor strains to mimic what may occur during a mixed infection *in vivo*. The co-cultures were sampled at four, six, eight, and ten hours post-inoculation and cultured on selective agar for transformants. Treatment with DNase I was used to ensure that transformation occurred during broth co-culture and not on the solid media after plating. In all experiments, piliation was lost by the donor strain over time, but approximately eighty percent of isolated colonies maintained piliated colony morphology. In co-cultures with AK1, *recA-* and OP100 bacteria, Cm^R, Cip^R transformants were isolated after ten hours of co-culture, and colonies appeared on selective plates after 48 hours of incubation (Table 9). Transformants were passed once to selective agar to verify stable transfer of the antibiotic marker. Co-cultures of FA19 *penA41, recA-* and OP100 bacteria yielded Cm^R, Cro^R transformants after only six hours of incubation and the transformants were clearly visible on the selective agar plates after 24 hours of incubation (Table 10). Similarly, Cm^R, Erm^R transformants were isolated after 6 hours of incubation of co-cultures containing KH15 and OP100 bacteria, and the transformants were visible on selective agar after 24 hours (Table 11). For all experiments, transformants were passed once on the appropriate selective agar to verify stable transfer of the antibiotic resistance marker, and *in vitro* transfer of the mutant allele in 3-5 transformants was confirmed by PCR and sequencing (Figures 20, 21 and 22). The efficiency of transfer was similar for the *mtrR$_{-79}$* and *penA41* alleles; transfer of *gyrA$_{91/95}$* was less efficient. This result is in contrast to results with gDNA in which transformation of *mtrR$_{-79}$* was less efficient. This may be a result of less robust growth of the donor strain AK1 *recA-* during competitive co-culture.

We conclude that transfer of these three resistance alleles can occur during co-culture of resistant and sensitive bacteria.

Figure 20. Sequence confirmation of the *gyrA*$_{91/95}$ mutation from AK1 in a Cm^R, Cip^R transformant isolated from a co-culture of OP100 and AK1, *recA*- bacteria.

Sequences are aligned to recipient strain OP100, which has the wild-type *gyrA* allele using the NCBI BLASTn suite. The nucleotide differences in the AK1, *recA*- and a Cm^R, Cip^R transformant (OP100 *gyrA*$_{91/95}$) encode for amino acid changes in the quinolone resistance determining region of GyrA (84), which carries the *gyrA*$_{91/95}$ mutation .

```
OP100 181 TCGTCGGCGACGTCATCGGTAAATACCACCCCCACGGCGATTCCGCAGTTTACGACACCA
AK1, recA-
      181 .......................................CT..........A......
OP100 gyrA91/95
      181 .......................................CT..........A......
```

Figure 21. Sequence confirmation of the mosaic *penA41* allele in a Cm^R, Cro^R
transformant isolated from a co-culture of OP100 and FA19 *penA41*, *recA-*
bacteria.

Sequences are aligned to recipient strain OP100, which has the wild-type *penA*
allele using the NCBI BLASTn suite. The portion of the gene shown here corresponds to
the region of the *penA41* mosaic allele that has been determined to carry the mutations
critical to ceftriaxone resistance (155).

```
OP100 241   GTAACCGTTTGCAGTCGGCTCGTCAATGGTTACCGCCACAATCACACGCGGATATTTTAG
FA19 penA41, recA-
      290   A..G...GG........T.....G.....................G..G.-....G.
OP100 penA41
      241   A..G...GG........T.....G.....................G..G.-....G.


301   CCGGGGCAAAACCGATGAAAGTGGCAACGTGTTTGTAATCGACGTAACGACCGTTAACCA
349   .G..............A..G..A..G.........TG...G.A.....C.....G..G.
301   .G..............A..G..A..G.........TG...G.A.....C.....G..G.


361   ACTTACGCGCCGTACCGGTTTTTGCGCCGACGTCGAAACCATCTACCGCACCGGCAATAC
409   ....G........G.......A........A........G..C........C..CG...
361   ....G........G.......A........A........G..C........C..CG...


421   CGCTACCGCC-GGCTTCAGTAACGGAAACCATCAACTCGCGCACtttttttGCAGTAGAG
469   ..G.G.....C...-..G..T.............GA.T...T..C.CGCGC..G..C..T
421   ..G.G.....C...-..G..T.............GA.T...T..C.CGCGC..G..C..T


480   GCTTTGATGACGCGTTTGCCTTTAGGGGCAACTGCTTGTTTTTCAAAGCTGACCGGCAAC
528   T......AT.T...........GC..C.....C..C..C..C.........G.....G.
481   T......AT.T...........GC..C.....C..C..C..C.........G.....G.


540   AATTCGCCGTCATGGGTCAAGACAGTATAGGCACGTGCCCAATTGCAACAGGCTTAATTG
588   ..AA.......G..C....GTG.G........G..C.-.........G......C.....
541   ..AA.......G..C....GTG.G........G..C.-.........G......C.....


600   CAGGCCATAACCCGAAAGACATCGTCGCCTGTTCGATAGGCCGCCATTTCCGCCAATTAC
647   ...A..G...-............................G.........CC.G........T.
602   ...A..G...-............................G.........CC.G........T.


660   GCAACACGCCCGCGCTTTCCCCGGGAAAGCCTGAATGCATGCGCACGCC-ACACACCTAA
706   T.....AA..T..AG.......C........C..G.....A.....A..G.TG.-..-..
663   T.....AA..T..AG......C........C..G.....A.....A..G.TG.-..-..


719   ATCGTGATAGAAATCGTACATTTCTT
764   T..A.......G..A..........
724   T..A.......G..A..........
```

Figure 22. Sequence confirmation of the *mtrR₋₇₉* allele in a Cm^R, Erm^R transformant isolated from a co-culture of OP100 and KH15, *recA-* bacteria.

Sequences are aligned to recipient strain OP100, which has the wild-type *mtr* locus using the NCBI BLASTn suite. The single nucleotide deletion in KH15, *recA-* and a Cm^R, Erm^R transformant (OP100 *mtrR₋₇₉*) is in the *mtrR* promoter region.

```
OP100 131   GTTTCATTATACATACACGATTGCACGGATAAAAAGTCTTTTTTATAATCCGCCCTCGTC
KH15, recA-
      144   ...............................‾............................
OP100, mtrR₋₇₉
      121   ...............................‾............................
```

Table 9. *In vitro* transfer of $gyrA_{91/95}$ during co-culture of OP100 and AK1 *recA-*
bacteria

Time (hours)	Growth on Cm + Cip (24 hrs)	Growth on Cm + Cip (48 hrs)
0	-	-
6	-	+
8	-	+
10	-	+

Results are representative of three independent experiments.
"+" indicates between 10-100 colonies.

Table 10. *In vitro* transfer of *penA41* during co-culture of OP100 and FA19 *penA41*, *recA-* bacteria

Time (hours)	Growth on Cm + Cro (24 hrs)	Growth on Cm + Cro (48 hrs)
0	-	-
6	++	++
8	++	++
10	++	++

Results are representative of three independent experiments.

"++" indicates greater than 100-500 colonies per 100 μL of co-culture.

Table 11. *In vitro* transfer of *mtrR$_{-79}$* during co-culture of OP100 and KH15 *recA-* bacteria

Time (hours)	Growth on Cm + Erm (24 hrs)	Growth on Cm + Erm (48 hrs)
0	-	-
6	++	++
8	++	++
10	++	++

Results are representative of three independent experiments.
 "++" indicates greater than 100-500 colonies per 100 µL of co-culture.

3.2c Antibiotic Resistance Determinants Were not Transferred *in vivo*

The transfer of antibiotic resistance between pathogenic strains of Gc has not been demonstrated *in vivo.* We used the murine model of infection as a surrogate for co-infection with a sensitive and resistant strain of Gc. Mice were inoculated with similar numbers of mostly piliated recipient strain OP100 and donor strains AK1 *recA-*, KH15 *recA-* or FA19 *penA41, recA-* on day zero. The swab suspension (500 µL) was incubated with DNase I, and we then quantitatively cultured vaginal swabs for seven consecutive days onto selective and non-selective media to measure colonization of the donor and recipient strains. The remaining swab suspension (400 µL) was cultured on media that contained double selection (Cm + Cip, Cro, or Erm) to select for transformants. DNase I was used to ensure transformation did not occur after plating. No transformants were isolated from any of the mice over the course of the experiment; plates were examined for transformants at 24 and 48 hours before discarding.

Examination of the recovery of each strain over time showed that 100% of mice were colonized by both donor and recipient strains for 2-4 days post-inoculation. However, only 3-5 mice (43-55%) in each group were co-infected by day 4 or 5 after inoculation (Figure 23).

Further, in order for Gc to be transformed with DNA, the recipient bacteria must be piliated. Over the course of the *in vivo* infection, it became clear that in addition to the loss of co-infection over time, piliation of the recipient strain also decreased. Between 22%-43% of vaginal isolates of OP100 had a nonpiliated colony morphology by day 5 of infection (Figure 24). This result stands in direct contrast to the *in vitro* experiments, where 80% of the recipient strain visually maintained a piliated colony morphology over the duration of the experiment, and resistance determinants were transferred between

strains (Tables 9-11). While we were unable to demonstrate the transfer of resistance *in vivo,* we conclude that this may be a result of both the insufficient amount of time that mice remained co-infected during infection and the loss of piliation by the recipient strain over the course of infection.

Figure 23. Duration of colonization by the donor and recipient strains in co-infection experiments

Mice were inoculated with similar numbers of donor and recipient bacteria. Percentage of mice colonized with the donor strain (black), recipient strain (blue) bacteria, or both strains (red) is plotted over time. Co-infection experiment with OP100 and AK1, *recA*- is shown in (A). Co-infection experiment with OP100 and FA19 *penA41, recA*-is shown in (B). Co-infection experiment with OP100 and KH15, *recA*- is shown in (C). The percent of co-infected mice in panel C (red line) overlays the percent of mice colonized by the donor strain. All data are a combination of two experiments, with a total sample size between seven and nine mice.

A

B

C

Inoculum:
KH15, *recA-*: 1.9x10^6
OP100: 1.2x10^6
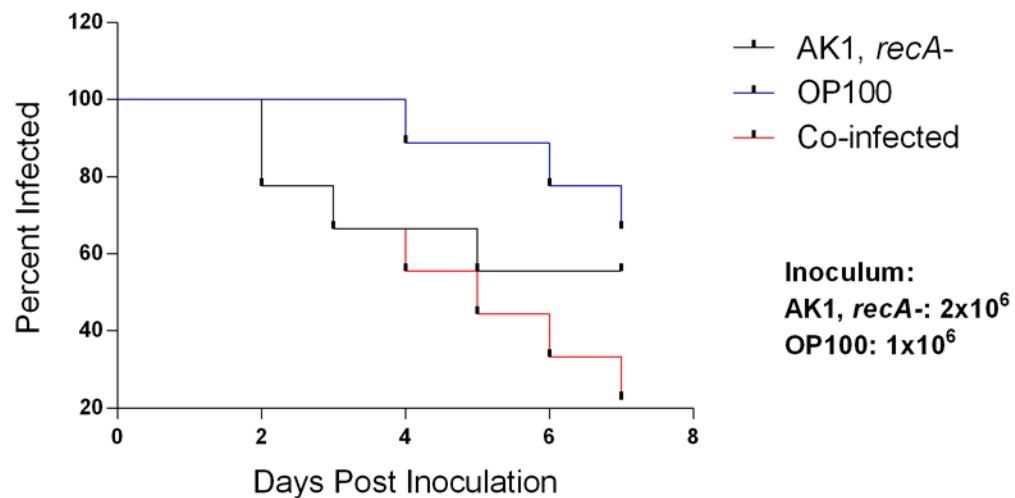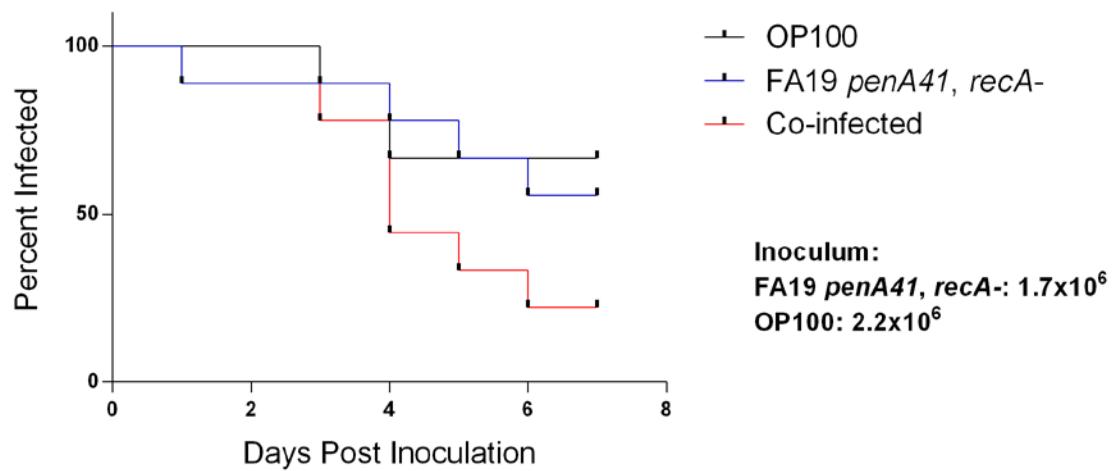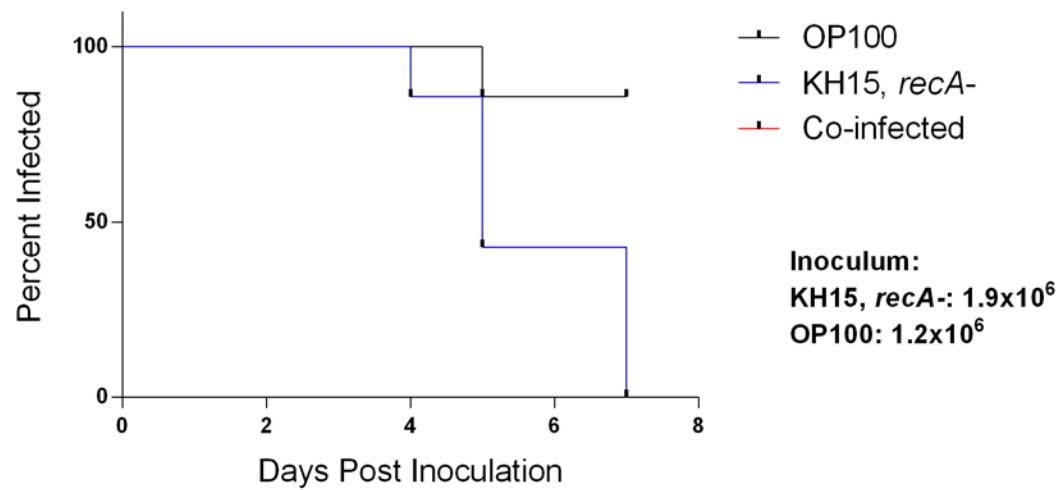
Figure 24. Piliation of recipient strain OP100 during co-infection experiments.

Mice were inoculated with similar numbers of donor and recipient bacteria. The percent of isolates of OP100 (Cm^R colonies that did not grow on double selection) with piliated colony morphology is shown over time during co-infection of OP100 and AK1, *recA-* (A), co-infection of OP100 and FA19 *penA41, recA-* (B), and during co-infection of OP100 and KH15, *recA-* (C). All data shown are a combination of two experiments, with a total sample size between seven and nine mice.

A
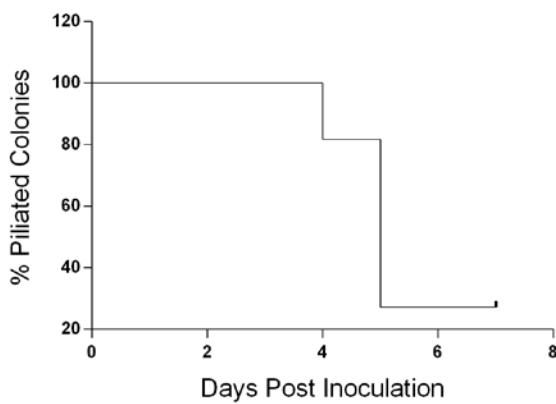
B
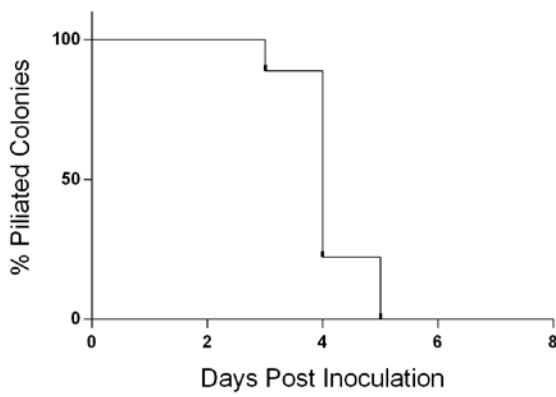
C
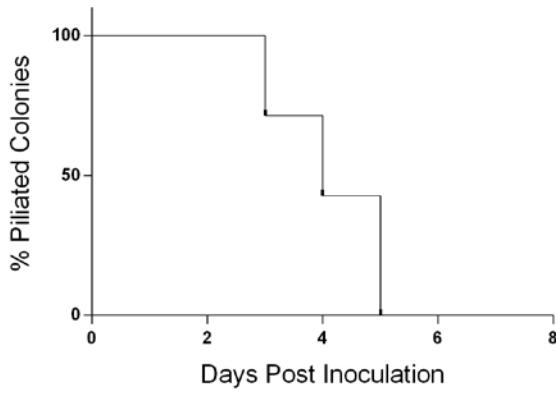
CHAPTER 4: Discussion

4.1 PREFACE

There are estimated to be 106 million Gc infections each year, 300,000 of which are reported in the US alone (26; 108). It is likely that the actual number of infections is much higher. Morbidity can be severe, particularly when the disease manifests initially as asymptomatic infection, which precludes treatment and allows spread to ascending infections (43; 75; 167; 168). The absence of a gonorrhea vaccine limits public health measures for controlling disease to antibiotic treatment. These strategies are relatively ineffective and are challenged by the genetic plasticity of this organism, which has led to the fear of untreatable gonorrhea as well as to the global spread of antibiotic resistance through horizontal genetic exchange.

This work examined two fundamental aspects of antibiotic resistance in Gc. The first study examined two newly emerged mutant *penA* alleles that confer resistance to the ESCs for their impact on microbial fitness *in vitro* and *in vivo*. Furthermore, we sought to determine whether selection for compensatory mutations occurs that allows ESC resistance to be maintained without compromising fitness. The significance of this question is that ESC antibiotics were only recently removed as the last remaining monotherapy for gonorrhea and are included in the currently recommended dual therapy for gonorrhea. Whether ESC resistance is likely to spread can be predicted from fitness studies comparing ESC^R and ESC^S strains. The second project was to test whether these mutant *penA* alleles can be transferred to a susceptible Gc strain during infection. The significance of this question stems from the fact that transfer of resistance alleles to Gc by natural transformation is routinely performed in the laboratory; it has never been demonstrated in an *in vivo* infection model. Evidence that *in vivo* transfer can indeed

138

occur would substantiate the theory that the spread of resistance in Gc occurs through uptake of linear DNA by susceptible strains during mixed infection with a resistant strain. We also tested two other resistance alleles that, unlike the mosaic *penA* alleles, increase Gc fitness *in vivo*. We hypothesized that these resistant transformants would be more likely to proliferate *in vivo*.

4.2 SPECIFIC AIM 1

Ceftriaxone is a member of the ESCs, a class of β-lactam antibiotics that are resistant to β-lactamase activity and kill bacteria by blocking the transpeptidases necessary for cell-wall synthesis. Here, we report that the ceftriaxone resistance-encoding mosaic *penA* alleles from clinical isolates H041 and F89 cause reduced fitness both *in vitro* and *in vivo*. The fitness defect was most evident for the *penA89* allele; we were unable to culture any strain FA19 *penA89* bacteria past day three of experimental murine infection. While there have been isolated cases of ceftriaxone treatment failures worldwide, high-level resistance has yet to spread (53; 93; 116; 158). It has only been seven years since the first ceftriaxone-resistant strain of Gc was reported, and most resistance in Gc takes fifteen to twenty years to spread globally (116). It is likely that we are simply at the front-end of the worldwide dissemination of these strains.

Our work, which examined isogenic mutant strains carrying mosaic *penA41* or *penA89* alleles with no other antibiotic resistance determinants, shows that mutations in the *penA* allele cause a clear fitness defect. The fitness defect was most pronounced in strains carrying the *penA89* allele, which is likely because the Ala-501 mutation in this strain confers a high fitness cost (153). From these observations, one might predict that strains bearing these alleles will not spread. However, as we hypothesized, compensatory

139

mutations arose that alleviated this fitness defect in strain FA19 *penA41*. In three independent experiments, four putative compensatory mutant strains were isolated. We characterized these mutant strains by first confirming that they conferred increased fitness against the Cro^R parent strain and then by testing whether they could also outcompete the Cro^S parent strain. Competition studies *in vitro* showed that two of the mutant strains, LV41A and LV41E had a 6.8-16.4-fold fitness advantage over the Cro^R parent; the other two showed a lesser (1.8-fold) competitive advantage. In contrast, all four compensatory mutant strains showed a strong competitive advantage over the Cro^R parent *in vivo*, with CIs ranging from 277- to 871-fold more fit. The four compensatory mutant strains exhibited equal fitness or a competitive disadvantage (1.7- and 5-fold reduced fitness) when cultured with the Cro^S parent strain FA19 *rpsL in vitro*. When co-inoculated into mice, three of the compensatory mutant strains out-competed the Cro^S strain by 2- to 3.6-fold. The remaining mutant strain, LV41B, was 2.5-fold less fit than strain FA19 *rpsL in vivo*.

As had been found for other compensatory mutations in β-lactam resistant mutant strains, attempts to demonstrate a phenotypic difference in the mutants that was related to bacterial division or peptidoglycan composition were unsuccessful (4). Whole genome sequencing was more fruitful with 1-3 mutations not related to *pilE* identified in each compensatory mutant strain. Interestingly, three of these four mutant strains have mutations in metabolic genes.

One of these mutations, *acnB*$_{G348D}$, was shown to confer a fitness advantage in mutant strain LV41C. The gene *acnB* encodes an aconitase, a metabolic enzyme critical to glucose metabolism. Glucose is a key source of energy for Gc, and the only

carbohydrate it can use for fermentation. Its utilization can be subdivided into three stages: glycolysis, which yields lactic acid, the formation of pyruvic acid from lactic acid, and the oxidation of pyruvic acid to yield carbon dioxide and acetate (105). Gc catabolizes glucose by means of both the Entner Doudoroff (ED) pathway and the Pentose-5-Phosphate (PP) pathway. Radiorespirometry studies demonstrated that the majority of the glucose was catabolized by the ED pathway (Fig. 25) (127). All of the enzymes necessary to oxidize acetate during aerobic respiration with a functioning tricarboxylic acid (TCA) cycle are present in Gc. However, many are not functional in the presence of glucose, with the exception of malate dehydrogenase. When the glucose stores of the bacterium have been depleted, the accumulated acetate of the ED and PP pathways is catabolized through the TCA (Fig. 26) (62).

Aconitase is a key metabolic enzyme within the TCA cycle. It catalyzes the oxidation of citrate to the intermediate *cis*-aconitate, and the conversion of *cis*-aconitate to isocitrate (Figure 26). Additionally, it is active in the 2-methylcitrate pathway, where it catalyzes the conversion of 2-methylcitrate to 2-methyl-*cis*-aconitate, and 2-methyl-*cis*-aconitate to methylisocitrate (103). This enzyme has no detectable activity when glucose is present, but after activation of the TCA cycle upon the depletion of glucose or entry into the stationary phase, aconitase activity is detectable (62; 127).

Figure 25. Carbohydrate metabolism in *Neisseria gonorrhoeae.*

Gc is only able to catabolize glucose, although it likely makes other carbohydrates. Glucose metabolism occurs via the Entner-Doudoroff pathway, with small amounts also catabolized through the pentose phosphate pathway (127). The net result of this catabolism is the accumulation of acetate, requiring further metabolism (127). Figure adapted from Chapter 11 by Morse *et al.* in Roberts' "The Gonococcus" (127).
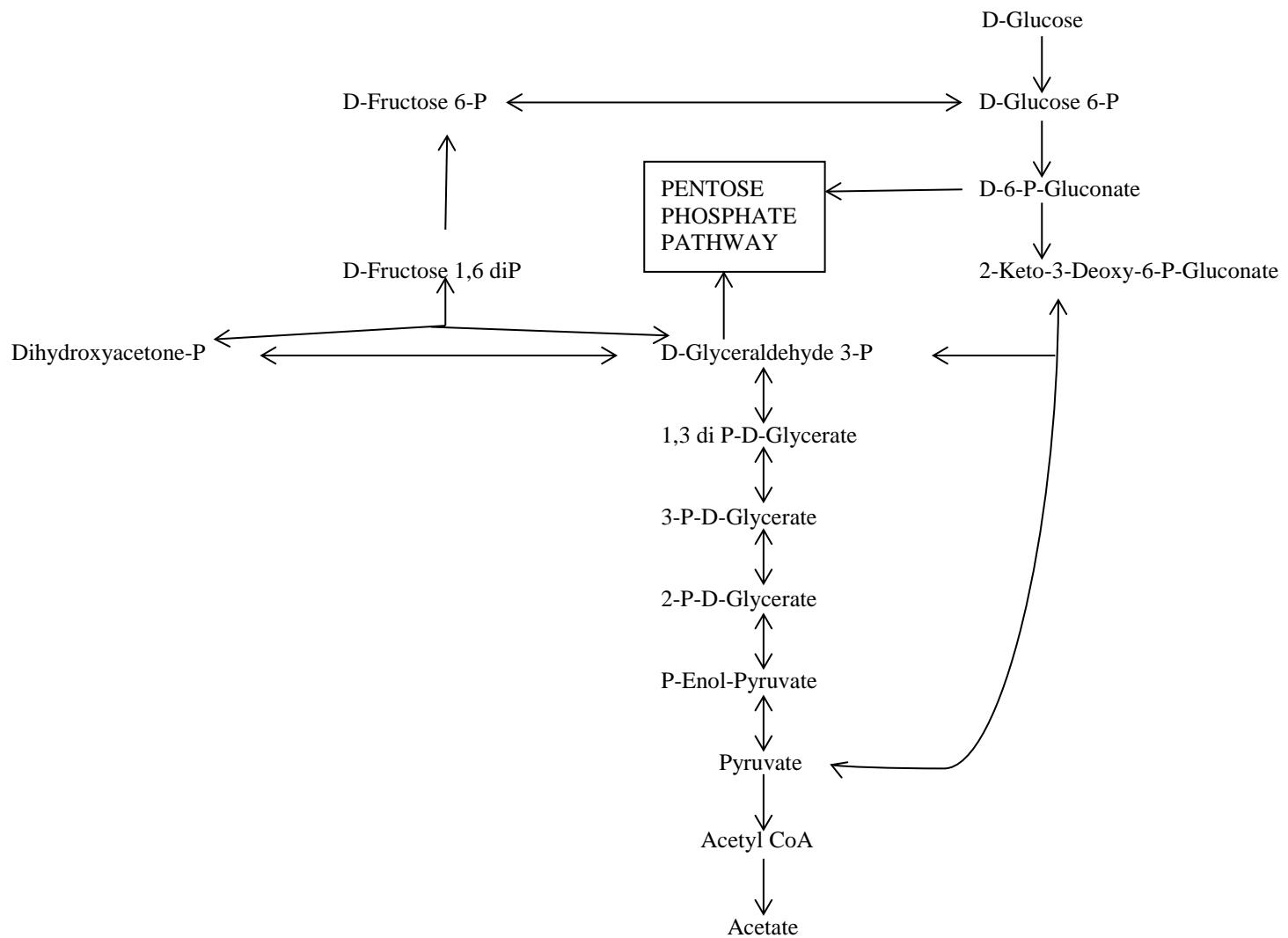
Figure 26. Acetate metabolism and the tricarboxylic acid cycle in *Neisseria gonorrhoeae.*

       The accrual of acetate during glucose catabolism is of particular importance in Gc, because *in vitro,* the presence of glucose in medium inhibits acetate catabolism and functioning of the TCA cycle (62; 127). Within this figure, solid arrows indicate reactions that can occur in the presence of lactate and glucose, while dashed arrows indicate reactions that can only happen after glucose or lactate exhaustion. This figure is modified from Chapter 11 by Morse *et al.* in Roberts' "The Gonococcus" (127).

P-ENOL-PYRUVATE

Pyruvate
kinase

PYRUVATE

Acetyl CoA
hydrolase

ACETATE

Acetyl
CoA

ACETYL CoA

PEP
carboxylase

Citrate Synthase

Malic
enzyme

OXALOACETATE

CITRATE

Malate
oxidase

Aconitase

L-MALATE

cis-ACONITATE

Fumarase

Aconitase

FUMARATE

L-ASPARTATE

ISOCITRATE

Succinate
dehydrogenase

Aspartate
Aminotransferase

Isocitrate
dehydrogenase

SUCCINATE

L-GLUTAMATE

OXALOSUCCINATE

2-ketoglutarate
dehydrogenase

Glutamate
dehydrogenase

Isocitrate
dehydrogenase

SUCCINYL CoA

2-KETOGLUTARATE

2-ketoglutarate dehydrogenase

145

A rapid logarithmic phase of growth and significantly enhanced fitness *in vivo* but not *in vitro* characterize strain LV41C. Enzyme kinetic data show that the $G_{348}D$ mutation bi-directionally impairs enzymatic function, which would seem to select against fitness (R. Nicholas, personal communication). However, when we moved $acnB_{G348D}$ into both parent strain FA19 *penA41* and fitness-impaired FA19 *penA89,* acquisition of the mutated allele confirmed the fitness benefit observed in LV41C. Of note, while fitness was increased, the fitness levels were not identical to LV41C. This result may be because FA19 *penA41, acnB_{G348D}* does not carry the PROKKA_02121$_{R165fs}$ mutation also identified in LV41C. A potential role for PROKKA_02121$_{R165fs}$ was further supported when FA19 *rpsL* was competed against FA19 *penA41, acnB_{G348D} in vivo*. The mutant was not more fit than the Cro^S strain, whereas LV41C was more fit. This result indicates that perhaps the increased fitness of strain LV41C is a cumulative effect of both the $acnB_{G348D}$ mutation and the PROKKA_02121$_{R165fs}$ mutation. It is worth noting that PROKKA_02121 is predicted to encode a hypothetical protein related to glyocsyl-tranferase-1, another metabolic protein.

Studies examining *Helicobacter pylori* aconitase have shown that it functions as a pleiotropic post-transcriptional regulator (12). Aconitase-mediated post-transcriptional regulation of the peptidoglycan deacetylase *pdgA* in *H. pylori* in response to oxidative stress and iron availability has been extensively studied (11). Aconitase is a Fe-S enzyme, and in *H. pylori,* the Fe-S cluster is disassembled under iron-limited conditions to give apo-aconitase. Apo-aconitase functions as a post-transcriptional regulator by binding the 3' untranslated region of *pdgA* to stabilize the transcript, which leads to increased expression. In an iron-limited environment, *pdgA* contributes to peptidoglycan lysozyme

146

resistance and immune evasion (12). While we have not demonstrated that gonococcal AcnB acts as a post-transcriptional regulator here, it is possible that AcnB may regulate peptidoglycan stability similarly by stabilizing *pdgA* transcripts or that of other cell wall biosynthesis genes. This hypothesis could explain how the *acnB* mutation compensates for the fitness defect conferred by the mosaic *penA* alleles in both FA19 *penA41* and FA19 *penA89*. To demonstrate this second activity for AcnB *in vitro* may require identifying culture conditions that mimic better the *in vivo* environment. We were unable to demonstrate that iron-limitation conferred a fitness advantage to these $acnB_{G348D}$ mutant strains. Other potential host stimuli could be reduced oxygen.

The compensatory mutants LV41A and LV41B both carry a mutation in *mleN*, which is predicted to encode a malate/sodium-lactate antiporter. MleN was first identified in the Gram-positive organism *Bacillus subtilis* and enhances growth on malate at low proton motive force and in certain physiological conditions, such as low oxygenation (172). Research performed using *Bacteroides succinogenes* showed that at a pH of less than 5.7, bacteria encounter a decrease in proton motive force (114; 131). Interestingly, studies have determined the vagina to be a low oxygen and low pH environment. However, in the estradiol-treated mouse model, the vaginal pH can range from 5.8-7.2 (106). Therefore, we hypothesize that $mleN_{\Delta A467}$ in LV41A and LV41B improves the function of MleN to enhance growth in the low oxygen vaginal environment.

Mutant LV41E did not have any identifiable mutations in annotated genes, but carried a unique intergenic mutation. One possible explanation for the observed fitness increase in this strain is that this intergenic mutation may be in *cis*-regulatory elements (CREs). CREs are transcriptional regulators located in intergenic regions of non-coding

DNA. Research has shown that mutation of CREs can drive pathoadaptation characterized by a significant fitness advantage within a particular ecological niche (119).

4.2a Limitations

One of the major limitations of this study is lack of knowledge concerning the biological relevance of compensatory mutations selected in the mouse model for human infections. The $acnB_{G348D}$ mutation is not present in either H041 or F89 (personal communication, M. Unemo). This question can be addressed as more ESC-resistant strains are isolated. For example, compensatory mutations in the RNA-polymerase genes were selected in ten clinical isolates of multi-drug resistant (MDR) *Mycobacterium tuberculosis* and six laboratory-derived resistant strains when cultured under *in vitro* conditions. The researchers subsequently identified these compensatory mutations in 30% of the circulating MDR *M. tuberculosis* strains (31).

Limitations inherent to our surrogate model of lower genital tract infection also limit the ability to test questions about Gc antibiotic resistance in human infections. Much of the spread of mosaic *penA* alleles has been attributed to pharyngeal and rectal infections of Gc, where the pathogenic strains are able to mix freely with commensal strains of *Neisseria* present at the site of infection, leading to the horizontal gene transfer of resistance mutations (14; 152). A pharyngeal model of Gc infection would therefore be very useful for studies of fitness and compensation, but is currently not available.

There are many important similarities in the human and murine lower genital tracts. Both feature low oxygen tension, and in both cases, glucose and lactose are available for metabolism (74). However, there are also some physiological differences between the human and murine genital tract that may cause the identification of

mutations that compensate for fitness in mice but not in humans, and conversely, that may prevent us from detecting mutations that increase fitness during human infection. As discussed previously, the murine vagina is more neutral than the female vagina. The pH of the murine vagina is similar to human cervical pH, which is the primary colonization site in human female infections. Mice have no menstrual bleeding during their reproductive cycle, which in humans is a source of proteases, hemoglobin, antibodies and other serum factors that could possibly select for certain mutations. Additionally, the need to treat mice with antibiotics to promote long-term Gc infection reduces the types of commensal flora present, which could also influence the microenvironment to which Gc is exposed.

In addition to physiological restrictions, host restrictions exist for some colonization receptors, soluble complement regulatory proteins, and the iron-binding glycoproteins transferrin and lactoferrin. Gc acquires iron from both transferrin and lactoferrin through outer membrane receptors that do not recognize murine transferrin or lactoferrin (74). Gc mutant strains that do not express these receptors do not have a fitness disadvantage in mice, likely due to non-host restricted iron stores available in the female lower genital tract (72). How each of these host restrictions may influence compensatory evolution of Gc in humans and mice is not known.

4.2b Future Directions

The outcome of the study examining the relationship between acquisition of mosaic *penA* alleles and Gc fitness has highlighted several exciting new avenues of inquiry. The emergence of ceftriaxone-resistant *Neisseria gonorrhoeae* has raised the specter of the loss of our ability to use ESCs to treat gonorrhea with confidence. First, the

data presented here demonstrate that while acquisition of the mosaic *penA* alleles from ceftriaxone-resistant clinical isolates is accompanied by a steep fitness cost, compensatory mutations can be selected *in vivo* that abrogate that fitness defect. As the race between acquisition of antibiotic resistance and development of novel antibiotic treatments continues, these secondary mutations may be seen as potential future drug targets. In order to leverage this work as a translational approach to drug development, further characterization of the *acnB*$_{G348D}$, *mleN*$_{\Delta A467}$, and the select intergenic mutations is needed to determine their specific mechanism of compensation.

Second, several questions regarding the gonococcal aconitase are raised by this work. First, the activity of aconitase during stationary phase should be examined. We observed a steep decline in viable bacteria in all mutant strains carrying the *acnB*$_{G348D}$ mutation during the stationary phase of growth. Real-time RT-PCR could be used to examine aconitase expression at different stages of growth. Because *acnB* mutants appear to be functional knockouts, with no measureable enzyme activity, it would be beneficial to create an *acnB*$_{G348D}$ knockout mutant and test both the knockout mutant and strains carrying the *acnB*$_{G348D}$ mutation for both growth characteristics and fitness. New studies in *H. pylori* demonstrated that apo-aconitase acts as a post-transcriptional regulator of peptidoglycan deacetylase (*pdgA*) by binding to the 3' untranslated end of the transcript, resulting in increased expression (11). This is important, as PdgA confers lysozyme resistance and immune evasion. We grew *acnB* mutants in low iron conditions *in vitro*, but did not detect any fitness benefit. However, further testing of FA19 *penA41* and the *acnB*$_{G348D}$ mutant under conditions where peptidoglycan stability is challenged by lysozyme may help to elucidate whether this regulation is present in Gc, and to determine

150

if the $acnB_{G348D}$ mutation plays a role. We hypothesize that while the $acnB$ knockout mutant will not have TCA cycle function or regulatory function, the $acnB_{G348D}$ mutant will be a functional knockout for TCA cycle activity but will retain regulatory function.

Additional work in *H. pylori* determined that $\Delta acnB$ strains had an oxidative-stress sensitive phenotype (12). In a global proteomics study, at least three diverse RNA transcripts were shown to be subject to aconitase-mediated regulation during oxidative stress, including a nickel-sequestering protein, an alkyl hydroperoxide reductase, and a flagellum response regulator. This led the authors to conclude that $acnB$ acts as a pleiotropic post-transcriptional regulator, something that has not yet been examined to any extend in Gc. The possibility that Gc AcnB has regulatory activity could be most directly tested by comparing the transcriptomes of FA19 *penA41* and FA19 *penA41*, $acnB_{G348D}$ during *in vivo* and *in vitro* growth.

Finally, it is clear that the *acnB* mutation causes a shift in fitness of FA19 *penA41,* but not in FA19 *rpsL.* This difference may mean that the mutation is specific to stabilization of the fitness defect conferred by the mosaic *penA* allele. We could test this hypothesis by introducing the $acnB_{G348D}$ mutation into a strain of Gc that carries both the $gyrA_{91/95}$ and the *parC* mutation, which are known to cause a fitness defect that is not associated with the *penA* allele (84). If the aconitase does stabilize *pdgA* as it does in *H. pylori,* no increased fitness would accompany the $acnB_{G348D}$ mutation, and this hypothesis would be supported.

Another open research direction that stems from this work is the role of $mleN_{\Delta A467}$ in restoring fitness to mutant strain FA19 *penA41.* This mutation must first be moved from LV41A and LV41B into strains FA19 *penA41,* FA19 *rpsL,* and FA19 *penA89.* The

fitness profiles of these recombinant strains could then be examined through both *in vitro* and *in vivo* experiments. We hypothesize that the mutant MleN protein works to enhance growth in the low oxygen tension of the vaginal environment. Further studies using both *in vitro* characterization in specialized growth conditions and *in vivo* animal experiments are necessary to examine this hypothesis.

Finally, the intergenic mutations we identified in mutant strains LV41A, LV41B, LV41C and LV41E may be in *cis*-regulatory elements, or non-coding regions of DNA that regulate nearby genes through transcriptional control. Hughes *et al.* demonstrated that CREs could be identified using computational biology combined with RNAseq technology (66). This approach may be useful for identifying CREs in Gc, and in determining if the areas we have identified as possible compensatory mutations map to any of these regions.

4.3 SPECIFIC AIM 2

As discussed previously, horizontal exchange of antibiotic resistance markers in Gc can occur through the transfer of plasmids carrying resistance determinants. It is believed that resistance is also transferred *in vivo* through natural transformation, but this has not been demonstrated successfully for Gc. In 2013, Weyand *et al.* demonstrated successful *in vivo* transfer of resistance genes between susceptible and resistant strains of *N. macaque* in the macaque oropharynx (174). These studies were exciting because they showed the transfer of resistance *in vivo* in an animal model of *Neisseria* mucosal colonization.

It is likely that resistance genes can also be transferred between two different Gc strains. While it is believed that infection with more than one strain of Gc frequently

occurs, evidence thereof was largely anecdotal until 2003, when Martin and Ison used the molecular technique of *opa* typing to track unique *opa* profiles to demonstrate that mixed infections do occur *in vivo* (96). Evidence that intergenetic exchange of *opa* genes can occur during mixed infections was also detected by these researchers (95). However, although mixed infections occur, it has yet to be shown that they can lead to the spread of antibiotic resistance in an *in vivo* model using human-specific pathogenic strains of Gc.

We attempted to demonstrate that *in vivo* transfer from a resistant Gc strain to a sensitive Gc strain could occur during infection. In these studies, we analyzed three resistance genes for which the impact on fitness in the mouse model was known:

- Cro resistance: *penA* allele → decreased fitness *in vivo*
- Cip resistance: *gyrA$_{91/95}$* allele → increased fitness *in vivo*
- Erm resistance: *mtrR$_{-79}$* mutation → increased fitness *in vivo*

We hypothesized that mutations that increase fitness *in vivo* are more likely to spread. We first showed that each resistance mutation was transferred by transformation using gDNA from each resistant donor strain incubated with the recipient strain in broth cultures at varying concentrations. As low as 50 ng/mL of DNA was needed for efficient recombination of the mutant *penA41* and *gyrA$_{91/95}$* alleles. Transfer of the *mtrR$_{-79}$* allele required 50-fold more DNA. This difference may be explained by possible differences in the frequency of the DNA uptake sequence near these genes, which occurs approximately once every 1000 base pairs of DNA (54). This is of particular note, as the *mtrR$_{-79}$* mutation occurs in the intergenic region between the *mtrR* gene and the *mtrCDE* operon, and there is no uptake sequence in this intergenic region or within the flanking genes. We

then measured gene transfer when donor and recipient bacteria were cultured together to better mimic a mixed infection.

We determined that during *in vitro* co-culture, each respective resistance mutation was stably transformed into the recipient strain. We incubated the cultures for up to ten hours to ensure that DNA would be released through either OMVs or bacterial lysis (40). In all three cases, transfer of resistance occurred, however, the $gyrA_{91/95}$ allele did not transfer as efficiently. This may be due to the observed *in vitro* growth advantage of FA19 *rpsL* over AK1 (CI of 0.1) that is not observed in the *in vitro* competition of FA19 *rpsL* and KH15 (84). Additionally, the $gyrA_{91/95}$ colony phenotype is much smaller than wild-type colonies and it takes 48 hours to detect on solid growth media, which also speaks to a growth disadvantage (A. Jerse, unpublished data). The acquisition of resistance mutations was verified by PCR for each resistance marker and associated mutation(s).

Finally, we tested the horizontal gene transfer of antibiotic resistance *in vivo*. The piliation phenotype of the recipient strain OP100 was carefully monitored over the course of infection, and the strain remained mostly piliated through day four of infection for all strain mixtures. Additionally, between 44-85% of mice remained co-infected with both strains by day four of infection. However, transfer of resistance mutations between any of the donor strains and recipient strain was not detected. This result was surprising, particularly for the strain mixtures that contained either the $gyrA_{91/95}$ mutation or the $mtrR_{-79}$ mutation, both of which increased *in vivo* fitness in previous studies. When horizontal gene transfer was examined in the macaque model of surrogate *Neisseria* infection, it took 44 days after inoculation with bacteria to detect transfer of resistance

determinants (174). We conclude that the loss of piliation as well as possible fitness differences between the strain backgrounds of the donor and recipient strains may have prevented *in vivo* transfer of resistance from occurring within the timeframe of the experiment.

4.3a Limitations

As mentioned previously, fitness differences between the strain backgrounds of donor and recipient strain may account for the loss of co-infection over time, and subsequent lack of horizontal gene transfer. We chose the recipient strain, OP100, because it carried chloramphenicol resistance, which allowed for selective plating. This strain was constructed in a FA1090 background. The *recA* mutant donor strains were in an FA19 background. There may be heretofore-unrecognized fitness differences between the FA19 and FA1090 backgrounds, which could challenge the ability to maintain concurrent infections by these strains.

Piliation is critical to horizontal gene transfer and is a phase-variable phenotype. Keeping piliation "on" during infection is a known limitation of the murine model of Gc infection. Unlike human infection, the majority of Gc recovered from mice are nonpiliated after 4-5 days. The reason for this phenomenon is unknown, but is likely due to the absence of the pilus receptor in the mouse (74).

4.3b Future Directions

Continued optimization of the *in vivo* co-infection model is needed to fully test whether horizontal gene transfer of resistance determinants during mixed infections of Gc occurs. Our strategy would be to repeat the *in vivo* portion of this experiment using a strain with a locked "on" piliation phenotype. Strain FA1090 $pilE_{NV}$ $pilC1_{PL}$, constructed

155

by Dr. Hank Seifert's laboratory (Northwestern University) carries a mutation in the guanine nucleotide repeat region located next to *pilE* that lessons antigenic variation and a mutation in the poly(G) tract of *pilC1* that results in a locked phase-on phenotype (7). This strain, which is locked in both phase and antigenic variation into a piliation "on" phenotype, should allow us to examine transfer of resistance without the confounding factor of loss of piliation *in vivo*.

Additionally, using a higher dose of the donor strains in the *in vivo* experiments may also improve the chances of observing *in vivo* transfer of resistance alleles during infection. In our experiments, mice were inoculated with an equal mix of donor and recipient strains. Using a larger inoculum of the donor strain compared to the recipient strain may allow more efficient exchange of resistance determinants to occur.

Finally, we only tested whether *in vivo* transfer would occur in the absence of antibiotic pressure to drive selection of the transfer of resistance. Repeat experiments with a sub-lethal dose of the relevant antibiotic could be used to determine if resistance is transferred when treatment is insufficient to clear the infection, but antibiotic pressure is present. These experiments would first require *in vivo* dose-response data for erythromycin, ceftriaxone, and ciprofloxacin against the sensitive recipient strain.

4.4 CONCLUSION

Novel findings in this work include both the *in vitro* and *in vivo* demonstration that mosaic *penA* alleles are less fit than their non-mosaic counterparts. *In vivo,* compensatory mutations were readily selected for one of these alleles, *penA41*, that abrogate the fitness disadvantage. Mutations in one and potentially two metabolic genes were identified (*acnB*$_{G348D}$ and *mleN*$_{\Delta A467}$, respectively) that can restore fitness, though

the basis of this fitness advantage is not yet known. Differences *in vivo* and *in vitro* for the $acnB_{G348D}$ mutation suggest AcnB may have yet undiscovered function(s) that lead to the fitness advantage observed *in vivo*. This work opens the door to new routes of investigation in the area of Gc metabolism and physiology. *In vivo*–selected compensatory mutations such as those we identified may lead to persistence and spread of these strains globally, and the identification of compensatory mutations is necessary to better understand and combat this spread.

Finally, we showed that clinically relevant resistance determinants that lead to high-level ceftriaxone, ciprofloxacin, and erythromycin resistance could be transferred *in vitro* between pathogenic Gc strains. Transfer of these resistance determinants *in vivo* may show how resistant strains are able to disseminate internationally. While we were not able to demonstrate horizontal gene transfer *in vivo,* further optimization of the co-infection model may demonstrate that transfer of resistance determinants can occur during mixed Gc infections. Furthermore, a working model of transfer of resistance during mixed infection will allow researchers to test whether *in vivo* fitness advantages associated with particular alleles increase the frequency of successful transfer.

APPENDIX A

A.1 ALA501 MUTATIONS IN PENICILLIN-BINDING PROTEIN 2

A.1a Introduction

There are sequence differences among the mosaic alleles of *penA*. Gc strain 35/02, isolated in Sweden in 2002, carries a mosaic *penA* allele called *penA35* that is identical to that of the Cro[R] strain F89, with the exception of an alanine at position 501. Strain F89, unlike strain 35/02, carries an A501P mutation, which confers a 4-fold greater level of ceftriaxone resistance compared to that of strain 35/02 (93; 158; 179). Strain 35/02 exhibits intermediate levels of resistance to ceftriaxone and cefixime, and high-level resistance to penicillin (179). The objective of this work was to determine whether *in vitro* fitness differences occur in isogenic strains that carry various amino acid substitutions made at the Ala501 residue. In addition to the proline substitution found in F89, we examined replacement of the alanine at position 501 with threonine, arginine, serine, and valine (Table 12).

Table 12. Amino acid replacement and MICs of strains used in this study		
Strain	Amino Acid Replacement	Cro MIC
FA19 *penA35*	None	0.031 μg/mL
FA19 *penA35*$_{A501T}$	Threonine	0.063 μg/mL
FA19 *penA35*$_{A501R}$	Arginine	0.125 μg/mL
FA19 *penA35*$_{A501S}$	Serine	0.063 μg/mL
FA19 *penA35*$_{A501P}$	Proline	0.125 μg/mL
FA19 *penA35*$_{A501V}$	Valine	0.063 μg/mL

A.1b Methods

A.1b1 Bacterial Strains

Bacterial strains used were constructed by Robert Nicholas (University of North Carolina) and are shown in Table 12. The MIC of Cro against these strains was determined according to the CDC method of agar dilution, as described previously (25).

A.1b2 Measurement of Growth Kinetics

Bacterial colonies with nonpiliated morphology were harvested from solid GC agar plates containing Kellogg's supplements after 18-20 hours of incubation. Bacteria were subsequently inoculated into 35 mL of supplemented GC broth with 5mM $NaHCO_3$ to an initial optical density of 0.08 at 600 nm (OD_{600}). Bacteria were grown for 8 hours at 220 RPM and 37°C. Aliquots were taken every hour to determine the OD_{600} value, and every two hours for quantitative culture to determine the number of viable bacteria (CFU/mL) (153).

A.1b3 Competitive Co-culture

Similar numbers of FA19 (wild-type) bacteria and FA19 *penA35* mutant bacteria carrying different *penA35* alleles were inoculated into supplemented GC broth with 5mM $NaHCO_3$. Samples were taken every two hours and quantitatively cultured onto non-selective GC agar (total number of CFU/mL) and GC agar with either 0.016 µg/mL or 0.031 µg/mL of ceftriaxone for selection (number of mutant CFU/mL). A competitive index was determined at each time point, which is the ratio of mutant to wild-type bacteria divided by the ratio of mutant to wild-type bacteria at initial inoculation. The experiment was repeated to test reproducibility (153).

A.1c Results

We first determined the MICs to ceftriaxone for all strains to facilitate later

selection steps for competitive co-culture. We determined that 0.016 µg/mL would be

used to select for all strains carrying mutant *penA35* alleles during co-culture experiments

(Table 12). We then examined the growth kinetics of each strain cultured independently

in GC broth over an 8-hour duration. All strains carrying the wild type *penA35* allele or

mutant *penA35* alleles were attenuated with respect to strain FA19 (Figure 27A and 27B).

A modest logarithmic phase was followed by a plateau that may be attributed to cell

death, based on viable cell counts for these strains. When we further examined

differences in fitness through competitive co-culture, again, all strains carrying the wild-

type or mutant *penA* alleles were attenuated relative to the wild type strain FA19 (Figure

27C). There was an overall fitness defect for all strains that increased over time, but the

proline and arginine substitutions conferred the most pronounced fitness defect (>1000-

fold) at 8 hours of incubation, compared to valine (200-fold decrease), or threonine and

serine (25-fold decrease) substitutions (Figure 27C).

Figure 27. Ala501 mutations confer differential biological fitness.

Growth of FA19 *penA35* and FA19 *penA35* mutant bacteria carrying different *penA35*-Ala501 alleles in GC broth over time as measured by (A) change in optical density and (B) viable counts. (C) Competitive co-cultures of FA19 *penA35* bacteria versus each FA19 *penA35* mutants. Results are expressed as CIs as described in the Methods. CIs less than 1 indicate reduced fitness relative to parent FA19 (Figure is modified from (153)).

A
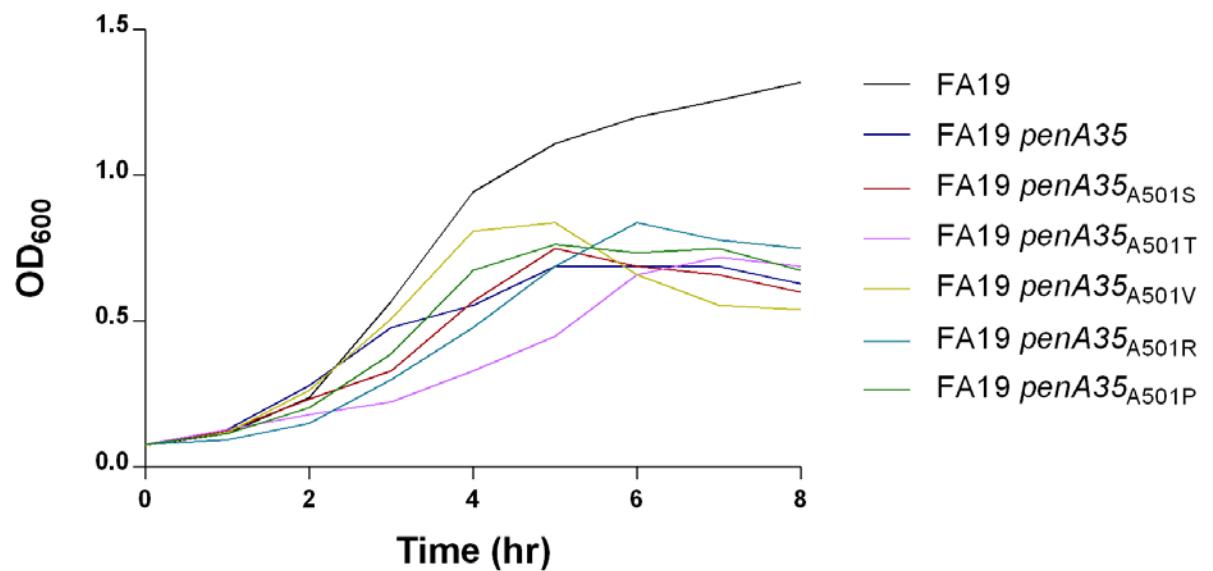
B
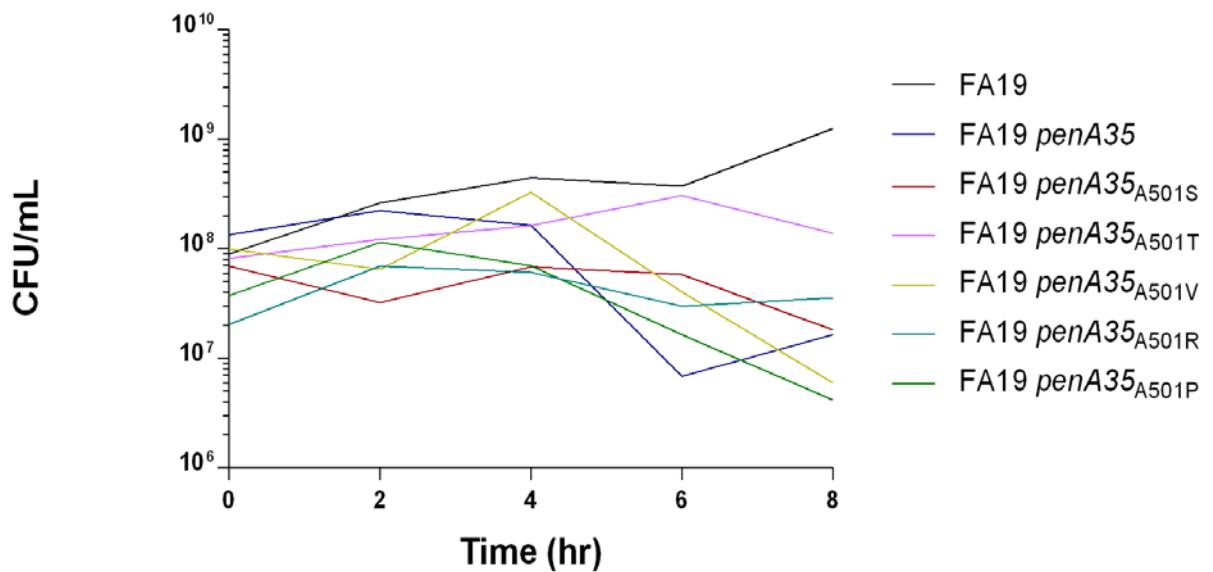
C
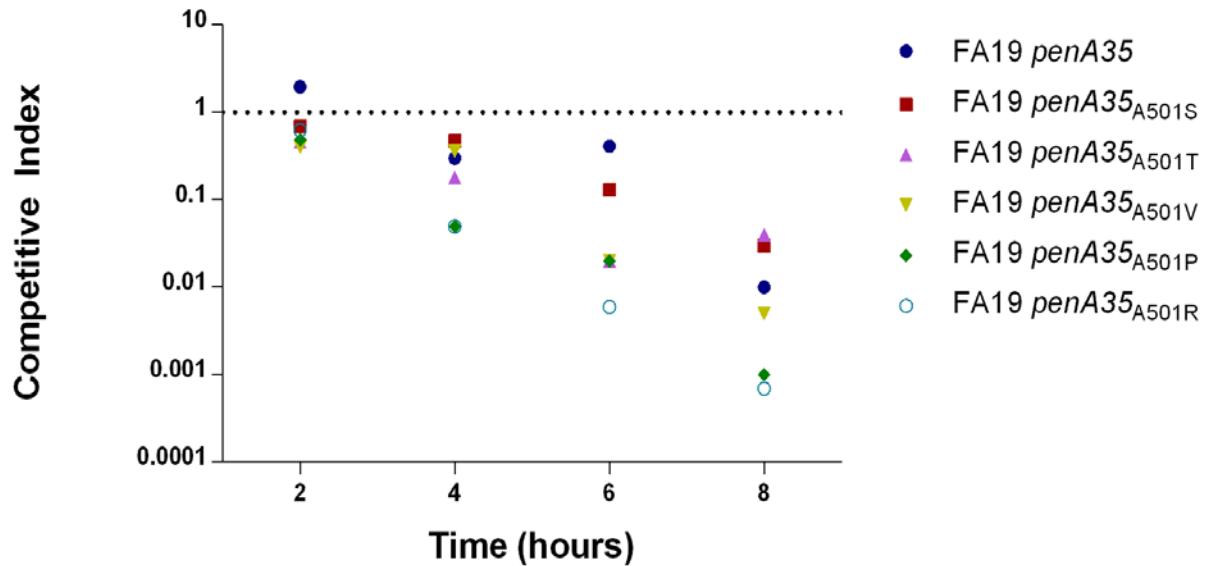
A.1d Conclusions

As expected, the mosaic *penA35* allele conferred a fitness defect to strain FA19.

We observed this defect while strains were grown independently in liquid growth

medium, as well as in competitive co-culture. This finding concurs with our previous data

that showed mosaic allele *penA41* and *penA89* conferred a strong fitness disadvantage *in*

vitro. Data from the competitive co-cultures clearly showed a gradient of effect when

different amino acids were substituted at the Ala501 location. The proline and the

arginine confer the greatest fitness disadvantage, perhaps because of the bulky side chain

that both of these amino acids carry, which may block access to the active site of the

enzyme.

REFERENCES

1.   http://www.ncbi.nlm.nih.gov/nuccore/NZ_ABZJ00000000.2
2.   Aas FE, Wolfgang M, Frye S, Dunham S, Lovold C, Koomey M. 2002.
        Competence for natural transformation in Neisseria gonorrhoeae: components of
        DNA binding and uptake linked to type IV pilus expression. *Molecular
        microbiology* 46:749-60
3.   Abrams AJ, Trees DL, Nicholas RA. 2015. Complete Genome Sequences of
        Three Neisseria gonorrhoeae Laboratory Reference Strains, Determined Using
        PacBio Single-Molecule Real-Time Technology. *Genome Announc* 3
4.   Albarracin Orio AG, Pinas GE, Cortes PR, Cian MB, Echenique J. 2011.
        Compensatory evolution of pbp mutations restores the fitness cost imposed by
        beta-lactam resistance in Streptococcus pneumoniae. *PLoS Pathog* 7:e1002000
5.   Ameyama S, Onodera S, Takahata M, Minami S, Maki N, et al. 2002. Mosaic-like
        structure of penicillin-binding protein 2 Gene (penA) in clinical isolates of
        Neisseria gonorrhoeae with reduced susceptibility to cefixime. *Antimicrob Agents
        Chemother* 46:3744-9
6.   Anderson JE, Hobbs MM, Biswas GD, Sparling PF. 2003. Opposing selective
        forces for expression of the gonococcal lactoferrin receptor. *Molecular
        microbiology* 48:1325-37
7.   Anderson MT, Dewenter L, Maier B, Seifert HS. 2014. Seminal plasma initiates a
        Neisseria gonorrhoeae transmission state. *MBio* 5:e01004-13
8.   Anderson MT, Seifert HS. 2011. Opportunity and means: horizontal gene transfer
        from the human host to a bacterial pathogen. *MBio* 2:e00005-11
9.   Andersson DI. 2003. Persistence of antibiotic resistant bacteria. *Curr Opin
        Microbiol* 6:452-6
10.   Antignac A, Rousselle JC, Namane A, Labigne A, Taha MK, Boneca IG. 2003.
        Detailed structural analysis of the peptidoglycan of the human pathogen Neisseria
        meningitidis. *J Biol Chem* 278:31521-8
11.   Austin CM, Maier RJ. 2013. Aconitase-mediated posttranscriptional regulation of
        Helicobacter pylori peptidoglycan deacetylase. *J Bacteriol* 195:5316-22
12.   Austin CM, Wang G, Maier RJ. 2015. Aconitase Functions as a Pleiotropic
        Posttranscriptional Regulator in Helicobacter pylori. *J Bacteriol* 197:3076-86
13.   Balthazar JT, Gusa A, Martin LE, Choudhury B, Carlson R, Shafer WM. 2011.
        Lipooligosaccharide Structure is an Important Determinant in the Resistance of
        Neisseria Gonorrhoeae to Antimicrobial Agents of Innate Host Defense. *Front
        Microbiol* 2:30
14.   Barry PM, Klausner JD. 2009. The use of cephalosporins for gonorrhea: the
        impending problem of resistance. *Expert Opin Pharmacother* 10:555-77
15.   Beck SC, Meyer TF. 2000. IgA1 protease from Neisseria gonorrhoeae inhibits
        TNFalpha-mediated apoptosis of human monocytic cells. *FEBS Lett* 472:287-92
16.   Belland RJ, Morrison SG, Ison C, Huang WM. 1994. Neisseria gonorrhoeae
        acquires mutations in analogous regions of gyrA and parC in fluoroquinolone-
        resistant isolates. *Molecular microbiology* 14:371-80

17. Berry JL, Cehovin A, McDowell MA, Lea SM, Pelicic V. 2013. Functional analysis of the interdependence between DNA uptake sequence and its cognate ComP receptor during natural transformation in Neisseria species. *PLoS Genet* 9:e1004014

18. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. 2015. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 13:42-51

19. Bramley J, Demarco de Hormaeche R, Constantinidou C, Nassif X, Parsons N, et al. 1995. A serum-sensitive, sialyltransferase-deficient mutant of Neisseria gonorrhoeae defective in conversion to serum resistance by CMP-NANA or blood cell extracts. *Microb Pathog* 18:187-95

20. Brannigan JA, Tirodimos IA, Zhang QY, Dowson CG, Spratt BG. 1990. Insertion of an extra amino acid is the main cause of the low affinity of penicillin-binding protein 2 in penicillin-resistant strains of Neisseria gonorrhoeae. *Molecular microbiology* 4:913-9

21. BroadInstitute. 2014. http://broadinstitute.github.io/picard.

22. Bush K, Courvalin P, Dantas G, Davies J, Eisenstein B, et al. 2011. Tackling antibiotic resistance. *Nat Rev Microbiol* 9:894-6

23. Cahoon LA, Seifert HS. 2011. Focusing homologous recombination: pilin antigenic variation in the pathogenic Neisseria. *Molecular microbiology* 81:1136-43

24. Camara J, Serra J, Ayats J, Bastida T, Carnicer-Pont D, et al. 2012. Molecular characterization of two high-level ceftriaxone-resistant Neisseria gonorrhoeae isolates detected in Catalonia, Spain. *J Antimicrob Chemother* 67:1858-60

25. CDC. 2005. *Neisseria gonorrhoeae* Reference Strains for Antimicrobial Susceptibility Testing.

26. CDC. 2013. *2013 Sexually Transmitted Diseases Surveillance: Gonorrhea*.

27. Centers for Disease C, Prevention. 2012. Update to CDC's Sexually transmitted diseases treatment guidelines, 2010: oral cephalosporins no longer a recommended treatment for gonococcal infections. *MMWR Morb Mortal Wkly Rep* 61:590-4

28. Chen I, Dubnau D. 2004. DNA uptake during bacterial transformation. *Nat Rev Microbiol* 2:241-9

29. Clavel F, Hance AJ. 2004. HIV drug resistance. *N Engl J Med* 350:1023-35

30. Cole JG, Fulcher NB, Jerse AE. 2010. Opacity proteins increase Neisseria gonorrhoeae fitness in the female genital tract due to a factor under ovarian control. *Infect Immun* 78:1629-41

31. Comas I, Borrell S, Roetzer A, Rose G, Malla B, et al. 2012. Whole-genome sequencing of rifampicin-resistant Mycobacterium tuberculosis strains identifies compensatory mutations in RNA polymerase genes. *Nat Genet* 44:106-10

32. Cooper MD, McGraw PA, Melly MA. 1986. Localization of gonococcal lipopolysaccharide and its relationship to toxic damage in human fallopian tube mucosa. *Infect Immun* 51:425-30

33. Cornelissen CN, Kelley M, Hobbs MM, Anderson JE, Cannon JG, et al. 1998. The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. *Molecular microbiology* 27:611-6

34. Courvalin P. 1994. Transfer of antibiotic resistance genes between gram-positive and gram-negative bacteria. *Antimicrob Agents Chemother* 38:1447-51

35. Cousin SL, Jr., Whittington WL, Roberts MC. 2003. Acquired macrolide resistance genes and the 1 bp deletion in the mtrR promoter in Neisseria gonorrhoeae. *J Antimicrob Chemother* 51:131-3

36. Criss AK, Kline KA, Seifert HS. 2005. The frequency and rate of pilin antigenic variation in Neisseria gonorrhoeae. *Molecular microbiology* 58:510-9

37. D'Ambrozio JA. 2015. *Insights into the Enhanced in vivo Fitness of Neisseria gonorrhoeae Driven by a Fluoroquinolone Resistance-Conferring Mutant DNA Gyrase*. The Uniformed Services University of the Health Sciences, Bethesda, MD. 106 pp.

38. Dahl KH, Mater DD, Flores MJ, Johnsen PJ, Midtvedt T, et al. 2007. Transfer of plasmid and chromosomal glycopeptide resistance determinants occurs more readily in the digestive tract of mice than in vitro and exconjugants can persist stably in vivo in the absence of glycopeptide selection. *J Antimicrob Chemother* 59:478-86

39. Ding Y, Onodera Y, Lee JC, Hooper DC. 2008. NorB, an efflux pump in Staphylococcus aureus strain MW2, contributes to bacterial fitness in abscesses. *J Bacteriol* 190:7123-9

40. Dorward DW, Garon CF, Judd RC. 1989. Export and intercellular transfer of DNA via membrane blebs of Neisseria gonorrhoeae. *J Bacteriol* 171:2499-505

41. Dowell D, Kirkcaldy RD. 2012. Effectiveness of gentamicin for gonorrhoea treatment: systematic review and meta-analysis. *Sex Transm Infect* 88:589-94

42. Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC. 2000. The tyrosine-86 allele of the pfmdr1 gene of Plasmodium falciparum is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. *Mol Biochem Parasitol* 108:13-23

43. Edwards JL, Apicella MA. 2004. The molecular mechanisms used by Neisseria gonorrhoeae to initiate infection differ between men and women. *Clin Microbiol Rev* 17:965-81, table of contents

44. Elkins C, Thomas CE, Seifert HS, Sparling PF. 1991. Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. *J Bacteriol* 173:3911-3

45. Engelkirk PG, Duben-Engelkirk JL. 2008. *Laboratory diagnosis of infectious diseases : essentials of diagnostic microbiology*. Baltimore: Wolters Kluwer Health/Lippincott Williams & Wilkins. xiv, 754 p. pp.

46. Faulstich M, Bottcher JP, Meyer TF, Fraunholz M, Rudel T. 2013. Pilus phase variation switches gonococcal adherence to invasion by caveolin-1-dependent host cell signaling. *PLoS Pathog* 9:e1003373

47. Fransen L, Nsanze H, D'Costa LJ, Brunham RC, Piot P. 1985. Parents of infants with ophthalmia neonatorum: a high-risk group for sexually transmitted diseases. *Sex Transm Dis* 12:150-4

48. Fransen L, Nsanze H, Klauss V, Van der Stuyft P, D'Costa L, et al. 1986. Ophthalmia neonatorum in Nairobi, Kenya: the roles of Neisseria gonorrhoeae and Chlamydia trachomatis. *The Journal of infectious diseases* 153:862-9

49. Garcia DL, Dillard JP. 2008. Mutations in ampG or ampD affect peptidoglycan fragment release from Neisseria gonorrhoeae. *J Bacteriol* 190:3799-807

50. Garvin LE, Bash MC, Keys C, Warner DM, Ram S, et al. 2008. Phenotypic and genotypic analyses of Neisseria gonorrhoeae isolates that express frequently recovered PorB PIA variable region types suggest that certain P1a porin sequences confer a selective advantage for urogenital tract infection. *Infect Immun* 76:3700-9

51. Geerts S, Gryseels B. 2000. Drug resistance in human helminths: current situation and lessons from livestock. *Clin Microbiol Rev* 13:207-22

52. Gilbert C, Bestman-Smith J, Boivin G. 2002. Resistance of herpesviruses to antiviral drugs: clinical impacts and molecular mechanisms. *Drug Resist Updat* 5:88-114

53. Golparian D, Ohlsson A, Janson H, Lidbrink P, Richtner T, et al. 2014. Four treatment failures of pharyngeal gonorrhoea with ceftriaxone (500 mg) or cefotaxime (500 mg), Sweden, 2013 and 2014. *Euro Surveill* 19

54. Goodman SD, Scocca JJ. 1991. Factors influencing the specific interaction of Neisseria gonorrhoeae with transforming DNA. *J Bacteriol* 173:5921-3

55. Gotschlich EC. 1994. Genetic locus for the biosynthesis of the variable portion of Neisseria gonorrhoeae lipooligosaccharide. *J Exp Med* 180:2181-90

56. Gould RG, Kane LW, Mueller JH. 1944. On the Growth Requirements of Neisseria Gonorrhoeae. *J Bacteriol* 47:287-92

57. Griffiss JM, Lammel CJ, Wang J, Dekker NP, Brooks GF. 1999. Neisseria gonorrhoeae coordinately uses Pili and Opa to activate HEC-1-B cell microvilli, which causes engulfment of the gonococci. *Infect Immun* 67:3469-80

58. Gunn JS, Stein DC. 1996. Use of a non-selective transformation technique to construct a multiply restriction/modification-deficient mutant of Neisseria gonorrhoeae. *Mol Gen Genet* 251:509-17

59. Haenni M, Moreillon P. 2008. Fitness cost and impaired survival in penicillin-resistant Streptococcus gordonii isolates selected in the laboratory. *Antimicrob Agents Chemother* 52:337-9

60. Hamilton HL, Dillard JP. 2006. Natural transformation of Neisseria gonorrhoeae: from DNA donation to homologous recombination. *Molecular microbiology* 59:376-85

61. Hauck CR, Meyer TF. 1997. The lysosomal/phagosomal membrane protein h-lamp-1 is a target of the IgA1 protease of Neisseria gonorrhoeae. *FEBS Lett* 405:86-90

62. Hebeler BH, Morse SA. 1976. Physiology and metabolism of pathogenic neisseria: tricarboxylic acid cycle activity in Neisseria gonorrhoeae. *J Bacteriol* 128:192-201

63. Higashi DL, Biais N, Weyand NJ, Agellon A, Sisko JL, et al. 2011. N. elongata produces type IV pili that mediate interspecies gene transfer with N. gonorrhoeae. *PLoS One* 6:e21373

64. Hobbs MM, Sparling PF, Cohen MS, Shafer WM, Deal CD, Jerse AE. 2011. Experimental Gonococcal Infection in Male Volunteers: Cumulative Experience with Neisseria gonorrhoeae Strains FA1090 and MS11mkC. *Front Microbiol* 2:123

65. Hu M, Nandi S, Davies C, Nicholas RA. 2005. High-level chromosomally mediated tetracycline resistance in Neisseria gonorrhoeae results from a point mutation in the rpsJ gene encoding ribosomal protein S10 in combination with the mtrR and penB resistance determinants. *Antimicrob Agents Chemother* 49:4327-34

66. Hughes JD, Estep PW, Tavazoie S, Church GM. 2000. Computational identification of cis-regulatory elements associated with groups of functionally related genes in Saccharomyces cerevisiae. *J Mol Biol* 296:1205-14

67. Ito M, Deguchi T, Mizutani KS, Yasuda M, Yokoi S, et al. 2005. Emergence and spread of Neisseria gonorrhoeae clinical isolates harboring mosaic-like structure of penicillin-binding protein 2 in Central Japan. *Antimicrob Agents Chemother* 49:137-43

68. James CE, Hudson AL, Davey MW. 2009. Drug resistance mechanisms in helminths: is it survival of the fittest? *Trends Parasitol* 25:328-35

69. James JF, Swanson J. 1978. Studies on gonococcus infection. XIII. Occurrence of color/opacity colonial variants in clinical cultures. *Infect Immun* 19:332-40

70. Jarvis GA, Chang TL. 2012. Modulation of HIV transmission by Neisseria gonorrhoeae: molecular and immunological aspects. *Curr HIV Res* 10:211-7

71. Jerse AE, Cohen MS, Drown PM, Whicker LG, Isbey SF, et al. 1994. Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. *J Exp Med* 179:911-20

72. Jerse AE, Crow ET, Bordner AN, Rahman I, Cornelissen CN, et al. 2002. Growth of Neisseria gonorrhoeae in the female mouse genital tract does not require the gonococcal transferrin or hemoglobin receptors and may be enhanced by commensal lactobacilli. *Infect Immun* 70:2549-58

73. Jerse AE, Wu H, Packiam M, Vonck RA, Begum AA, Garvin LE. Estradiol-Treated Female Mice as Surrogate Hosts for Neisseria gonorrhoeae Genital Tract Infections. *Front Microbiol* 2:107

74. Jerse AE, Wu H, Packiam M, Vonck RA, Begum AA, Garvin LE. 2011. Estradiol-Treated Female Mice as Surrogate Hosts for Neisseria gonorrhoeae Genital Tract Infections. *Front Microbiol* 2:107

75. John J, Donald WH. 1978. Asymptomatic urethral gonorrhoea in men. *Br J Vener Dis* 54:322-3

76. Johnson SR, Morse SA. 1988. Antibiotic resistance in Neisseria gonorrhoeae: genetics and mechanisms of resistance. *Sex Transm Dis* 15:217-24

77. Johnston DM, Cannon JG. 1999. Construction of mutant strains of Neisseria gonorrhoeae lacking new antibiotic resistance markers using a two gene cassette with positive and negative selection. *Gene* 236:179-84

78. Kandler JL, Joseph SJ, Balthazar JT, Dhulipala V, Read TD, et al. 2014. Phase-variable expression of lptA modulates the resistance of Neisseria gonorrhoeae to cationic antimicrobial peptides. *Antimicrob Agents Chemother* 58:4230-3

79. Kellogg DS, Jr., Peacock WL, Jr., Deacon WE, Brown L, Pirkle DI. 1963. Neisseria Gonorrhoeae. I. Virulence Genetically Linked to Clonal Variation. *J Bacteriol* 85:1274-9

80. Kerle KK, Mascola JR, Miller TA. 1992. Disseminated gonococcal infection. *Am Fam Physician* 45:209-14

81.   Kirkcaldy RD, Weinstock HS, Moore PC, Philip SS, Wiesenfeld HC, et al. 2014. The efficacy and safety of gentamicin plus azithromycin and gemifloxacin plus azithromycin as treatment of uncomplicated gonorrhea. *Clin Infect Dis* 59:1083-91

82.   Knapp JS, Clark VL. 1984. Anaerobic growth of Neisseria gonorrhoeae coupled to nitrite reduction. *Infect Immun* 46:176-81

83.   Kruger NJ, Stingl K. 2011. Two steps away from novelty--principles of bacterial DNA uptake. *Molecular microbiology* 80:860-7

84.   Kunz AN, Begum AA, Wu H, D'Ambrozio JA, Robinson JM, et al. 2012. Impact of fluoroquinolone resistance mutations on gonococcal fitness and in vivo selection for compensatory mutations. *The Journal of infectious diseases* 205:1821-9

85.   Lankford CE, Scott V, Cox MF, Cooke WR. 1943. Some Aspects of Nutritional Variation of the Gonococcus. *J Bacteriol* 45:321-7

86.   Leathwick DM. 2013. Managing anthelmintic resistance--parasite fitness, drug use strategy and the potential for reversion towards susceptibility. *Vet Parasitol* 198:145-53

87.   Lewis DA. 2010. The Gonococcus fights back: is this time a knock out? *Sex Transm Infect* 86:415-21

88.   Li H. https://samtools.github.io/bcftools/bcftools.html

89.   Li H. 2011. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics* 27:2987-93

90.   Li H, Durbin R. 2010. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 26:589-95

91.   Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, et al. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25:2078-9

92.   Lin L, Ayala P, Larson J, Mulks M, Fukuda M, et al. 1997. The Neisseria type 2 IgA1 protease cleaves LAMP1 and promotes survival of bacteria within epithelial cells. *Molecular microbiology* 24:1083-94

93.   Lindberg R, Fredlund H, Nicholas R, Unemo M. 2007. Neisseria gonorrhoeae isolates with reduced susceptibility to cefixime and ceftriaxone: association with genetic polymorphisms in penA, mtrR, porB1b, and ponA. *Antimicrob Agents Chemother* 51:2117-22

94.   Maness MJ, Sparling PF. 1973. Multiple antibiotic resistance due to a single mutation in Neisseria gonorrhoeae. *The Journal of infectious diseases* 128:321-30

95.   Martin IM, Ghani A, Bell G, Kinghorn G, Ison CA. 2003. Persistence of two genotypes of Neisseria gonorrhoeae during transmission. *J Clin Microbiol* 41:5609-14

96.   Martin IM, Ison CA. 2003. Detection of mixed infection of Neisseria gonorrhoeae. *Sex Transm Infect* 79:56-8

97.   Martinez JL, Baquero F. 2002. Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. *Clin Microbiol Rev* 15:647-79

98.   McClure R, Nudel K, Massari P, Tjaden B, Su X, et al. 2015. The Gonococcal Transcriptome during Infection of the Lower Genital Tract in Women. *PLoS One* 10:e0133982

99.   McGee ZA, Clemens CM, Jensen RL, Klein JJ, Barley LR, Gorby GL. 1992. Local induction of tumor necrosis factor as a molecular mechanism of mucosal damage by gonococci. *Microb Pathog* 12:333-41

100.   McGee ZA, Jensen RL, Clemens CM, Taylor-Robinson D, Johnson AP, Gregg CR. 1999. Gonococcal infection of human fallopian tube mucosa in organ culture: relationship of mucosal tissue TNF-alpha concentration to sloughing of ciliated cells. *Sex Transm Dis* 26:160-5

101.   Mehr IJ, Seifert HS. 1997. Random shuttle mutagenesis: gonococcal mutants deficient in pilin antigenic variation. *Molecular microbiology* 23:1121-31

102.   Menard R, Sansonetti PJ, Parsot C. 1993. Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of Shigella flexneri entry into epithelial cells. *J Bacteriol* 175:5899-906

103.   Miller WG, Parker CT, Rubenfield M, Mendz GL, Wosten MM, et al. 2007. The complete genome sequence and analysis of the epsilonproteobacterium Arcobacter butzleri. *PLoS One* 2:e1358

104.   Morse SA, Johnson SR, Biddle JW, Roberts MC. 1986. High-level tetracycline resistance in Neisseria gonorrhoeae is result of acquisition of streptococcal tetM determinant. *Antimicrob Agents Chemother* 30:664-70

105.   Morse SA, Stein S, Hines J. 1974. Glucose metabolism in Neisseria gonorrhoeae. *J Bacteriol* 120:702-14

106.   Muench DF, Kuch DJ, Wu H, Begum AA, Veit SJ, et al. 2009. Hydrogen peroxide-producing lactobacilli inhibit gonococci in vitro but not during experimental genital tract infection. *The Journal of infectious diseases* 199:1369-78

107.   Nandi S, Swanson S, Tomberg J, Nicholas RA. 2015. Diffusion of antibiotics through the PilQ secretin in Neisseria gonorrhoeae occurs through the immature, sodium dodecyl sulfate-labile form. *J Bacteriol* 197:1308-21

108.   Ndowa F, Lusti-Narasimhan M. 2012. Global action plan to control the spread and impact of antimicrobial resistance in Neisseria gonorrhoeae., World Health Organization

109.   Ndowa F, Lusti-Narasimhan M, Unemo M. 2012. The serious threat of multidrug-resistant and untreatable gonorrhoea: the pressing need for global action to control the spread of antimicrobial resistance, and mitigate the impact on sexual and reproductive health. *Sex Transm Infect* 88:317-8

110.   Nelson M. 1973. Uncomplicated male gonorrhea--a review. *Calif Med* 118:10-3

111.   Ng LK, Martin I, Liu G, Bryden L. 2002. Mutation in 23S rRNA associated with macrolide resistance in Neisseria gonorrhoeae. *Antimicrob Agents Chemother* 46:3020-5

112.   Ngampasutadol J, Ram S, Gulati S, Agarwal S, Li C, et al. 2008. Human factor H interacts selectively with Neisseria gonorrhoeae and results in species-specific complement evasion. *J Immunol* 180:3426-35

113. Nijhuis M, Schuurman R, de Jong D, Erickson J, Gustchina E, et al. 1999. Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. *AIDS* 13:2349-59

114. O'Hanlon DE, Moench TR, Cone RA. 2013. Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. *PLoS One* 8:e80074

115. O'Regan E, Quinn T, Frye JG, Pages JM, Porwollik S, et al. Fitness costs and stability of a high-level ciprofloxacin resistance phenotype in Salmonella enterica serotype enteritidis: reduced infectivity associated with decreased expression of Salmonella pathogenicity island 1 genes. *Antimicrob Agents Chemother* 54:367-74

116. Ohnishi M, Golparian D, Shimuta K, Saika T, Hoshina S, et al. 2011. Is Neisseria gonorrhoeae initiating a future era of untreatable gonorrhea?: detailed characterization of the first strain with high-level resistance to ceftriaxone. *Antimicrob Agents Chemother* 55:3538-45

117. Ohnishi M, Saika T, Hoshina S, Iwasaku K, Nakayama S, et al. 2011. Ceftriaxone-resistant Neisseria gonorrhoeae, Japan. *Emerg Infect Dis* 17:148-9

118. Olesky M, Hobbs M, Nicholas RA. 2002. Identification and analysis of amino acid mutations in porin IB that mediate intermediate-level resistance to penicillin and tetracycline in Neisseria gonorrhoeae. *Antimicrob Agents Chemother* 46:2811-20

119. Osborne SE, Walthers D, Tomljenovic AM, Mulder DT, Silphaduang U, et al. 2009. Pathogenic adaptation of intracellular bacteria by rewiring a cis-regulatory input function. *Proc Natl Acad Sci U S A* 106:3982-7

120. Pfaller MA. 2012. Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. *Am J Med* 125:S3-13

121. Quinn R. 2013. Rethinking antibiotic research and development: World War II and the penicillin collaborative. *Am J Public Health* 103:426-34

122. Ramsey ME, Woodhams KL, Dillard JP. 2011. The Gonococcal Genetic Island and Type IV Secretion in the Pathogenic Neisseria. *Front Microbiol* 2:61

123. Richman DD. 2006. Antiviral drug resistance. *Antiviral Res* 71:117-21

124. Richman DD, Morton SC, Wrin T, Hellmann N, Berry S, et al. 2004. The prevalence of antiretroviral drug resistance in the United States. *AIDS* 18:1393-401

125. Roberts MC. 1989. Plasmids of Neisseria gonorrhoeae and other Neisseria species. *Clin Microbiol Rev* 2 Suppl:S18-23

126. Roberts MC, Chung WO, Roe D, Xia M, Marquez C, et al. 1999. Erythromycin-resistant Neisseria gonorrhoeae and oral commensal Neisseria spp. carry known rRNA methylase genes. *Antimicrob Agents Chemother* 43:1367-72

127. Roberts RB. 1977. *The Gonococcus*. New York: Wiley. xii, 436 p. pp.

128. Roe E, Jones RI, Lowbury EJL. 1971. Transfer of Antibiotic Resistance Between Pseudomonas Aeruginosa, Eschericia Coli, and Other Gram-Negative Bacilli in Burns. *The Lancet* 297:149-52

129. Ropp PA, Hu M, Olesky M, Nicholas RA. 2002. Mutations in ponA, the gene encoding penicillin-binding protein 1, and a novel locus, penC, are required for high-level chromosomally mediated penicillin resistance in Neisseria gonorrhoeae. *Antimicrob Agents Chemother* 46:769-77

130.   Rosenthal PJ. 2013. The interplay between drug resistance and fitness in malaria parasites. *Molecular microbiology* 89:1025-38

131.   Russell JB. 1987. Effect of extracellular pH on growth and proton motive force of Bacteroides succinogenes, a cellulolytic ruminal bacterium. *Appl Environ Microbiol* 53:2379-83

132.   Sadarangani M, Pollard AJ, Gray-Owen SD. 2011. Opa proteins and CEACAMs: pathways of immune engagement for pathogenic Neisseria. *FEMS Microbiol Rev* 35:498-514

133.   Sangster N, Batterham P, Chapman HD, Duraisingh M, Le Jambre L, et al. 2002. Resistance to antiparasitic drugs: the role of molecular diagnosis. *Int J Parasitol* 32:637-53

134.   Sarubbi FA, Jr., Sparling PF. 1974. Transfer of antibiotic resistance in mixed cultures of Neisseria gonorrhoeae. *The Journal of infectious diseases* 130:660-3

135.   Schulz zur Wiesch P, Engelstadter J, Bonhoeffer S. 2010. Compensation of fitness costs and reversibility of antibiotic resistance mutations. *Antimicrob Agents Chemother* 54:2085-95

136.   Seifert HS, Ajioka RS, Marchal C, Sparling PF, So M. 1988. DNA transformation leads to pilin antigenic variation in Neisseria gonorrhoeae. *Nature* 336:392-5

137.   Seifert HS, Wright CJ, Jerse AE, Cohen MS, Cannon JG. 1994. Multiple gonococcal pilin antigenic variants are produced during experimental human infections. *J Clin Invest* 93:2744-9

138.   Shafer WM, Folster JP. 2006. Towards an understanding of chromosomally mediated penicillin resistance in Neisseria gonorrhoeae: evidence for a porin-efflux pump collaboration. *J Bacteriol* 188:2297-9

139.   Shaughnessy J, Ram S, Bhattacharjee A, Pedrosa J, Tran C, et al. 2011. Molecular characterization of the interaction between sialylated Neisseria gonorrhoeae and factor H. *J Biol Chem* 286:22235-42

140.   Shultz TR, Tapsall JW, White PA. 2001. Correlation of in vitro susceptibilities to newer quinolones of naturally occurring quinolone-resistant Neisseria gonorrhoeae strains with changes in GyrA and ParC. *Antimicrob Agents Chemother* 45:734-8

141.   Simms AN, Jerse AE. 2006. In vivo selection for Neisseria gonorrhoeae opacity protein expression in the absence of human carcinoembryonic antigen cell adhesion molecules. *Infect Immun* 74:2965-74

142.   Smith H, Parsons NJ, Cole JA. 1995. Sialylation of neisserial lipopolysaccharide: a major influence on pathogenicity. *Microb Pathog* 19:365-77

143.   Soler-Garcia AA. 2002. *Contribution of Neisseria gonorrhoeae Catalase in Defense Against Toxic Oxygen Radicals and Neutrophils, and Its Role During Experimental Genital Tract Infection of Mice* Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814 475 pp.

144.   Soler-Garcia AA, Jerse AE. 2004. A Neisseria gonorrhoeae catalase mutant is more sensitive to hydrogen peroxide and paraquat, an inducer of toxic oxygen radicals. *Microb Pathog* 37:55-63

145.   Sparling PF. 1966. Genetic transformation of Neisseria gonorrhoeae to streptomycin resistance. *J Bacteriol* 92:1364-71

146. Sparling PF, Modolell J, Takeda Y, Davis BD. 1968. Ribosomes from Escherichia coli merodiplods heterozygous for resistance to streptomycin and to spectinomycin. *J Mol Biol* 37:407-21

147. Spellberg B, Taylor-Blake B. 2013. On the exoneration of Dr. William H. Stewart: debunking an urban legend. *Infect Dis Poverty* 2:3

148. Spratt BG, Bowler LD, Zhang QY, Zhou J, Smith JM. 1992. Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal Neisseria species. *J Mol Evol* 34:115-25

149. Springer B, Kidan YG, Prammananan T, Ellrott K, Bottger EC, Sander P. 2001. Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance. *Antimicrob Agents Chemother* 45:2877-84

150. Stefanova ME, Tomberg J, Davies C, Nicholas RA, Gutheil WG. 2004. Overexpression and enzymatic characterization of Neisseria gonorrhoeae penicillin-binding protein 4. *Eur J Biochem* 271:23-32

151. Tanaka M, Sakuma S, Takahashi K, Nagahuzi T, Saika T, et al. 1998. Analysis of quinolone resistance mechanisms in Neisseria gonorrhoeae isolates in vitro. *Sex Transm Infect* 74:59-62

152. Tapsall JW, Ndowa F, Lewis DA, Unemo M. 2009. Meeting the public health challenge of multidrug- and extensively drug-resistant Neisseria gonorrhoeae. *Expert Rev Anti Infect Ther* 7:821-34

153. Tomberg J, Fedarovich A, Vincent L, Jerse AE, Unemo M, et al. 2016. Ala501 mutations in Penicillin-Binding Protein 2 from Neisseria gonorrhoeae: differential effects on resistance, transpeptidase activity, and biological fitness. *In preparation*

154. Tomberg J, Unemo M, Davies C, Nicholas RA. 2010. Molecular and structural analysis of mosaic variants of penicillin-binding protein 2 conferring decreased susceptibility to expanded-spectrum cephalosporins in Neisseria gonorrhoeae: role of epistatic mutations. *Biochemistry* 49:8062-70

155. Tomberg J, Unemo M, Ohnishi M, Davies C, Nicholas RA. 2013. Identification of amino acids conferring high-level resistance to expanded-spectrum cephalosporins in the penA gene from Neisseria gonorrhoeae strain H041. *Antimicrob Agents Chemother* 57:3029-36

156. Trzcinski K, Thompson CM, Gilbey AM, Dowson CG, Lipsitch M. 2006. Incremental increase in fitness cost with increased beta -lactam resistance in pneumococci evaluated by competition in an infant rat nasal colonization model. *J Infect Dis* 193:1296-303

157. Unemo M, Golparian D, Hellmark B. 2014. First three Neisseria gonorrhoeae isolates with high-level resistance to azithromycin in Sweden: a threat to currently available dual-antimicrobial regimens for treatment of gonorrhea? *Antimicrob Agents Chemother* 58:624-5

158. Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P. 2012. High-level cefixime- and ceftriaxone-resistant Neisseria gonorrhoeae in France: novel penA mosaic allele in a successful international clone causes treatment failure. *Antimicrob Agents Chemother* 56:1273-80

159. Unemo M, Golparian D, Skogen V, Olsen AO, Moi H, et al. 2013. Neisseria gonorrhoeae strain with high-level resistance to spectinomycin due to a novel

resistance mechanism (mutated ribosomal protein S5) verified in Norway. *Antimicrob Agents Chemother* 57:1057-61

160. Unemo M, Golparian D, Stary A, Eigentler A. 2011. First Neisseria gonorrhoeae strain with resistance to cefixime causing gonorrhoea treatment failure in Austria, 2011. *Euro Surveill* 16

161. Unemo M, Golparian D, Syversen G, Vestrheim DF, Moi H. 2010. Two cases of verified clinical failures using internationally recommended first-line cefixime for gonorrhoea treatment, Norway, 2010. *Euro Surveill* 15

162. Unemo M, Nicholas RA. 2012. Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. *Future Microbiol* 7:1401-22

163. Unemo M, Shafer WM. 2011. Antibiotic resistance in Neisseria gonorrhoeae: origin, evolution, and lessons learned for the future. *Ann N Y Acad Sci* 1230:E19-28

164. Upcroft P, Upcroft JA. 2001. Drug targets and mechanisms of resistance in the anaerobic protozoa. *Clin Microbiol Rev* 14:150-64

165. van der Woude MW, Baumler AJ. 2004. Phase and antigenic variation in bacteria. *Clin Microbiol Rev* 17:581-611, table of contents

166. Veal WL, Nicholas RA, Shafer WM. 2002. Overexpression of the MtrC-MtrD-MtrE efflux pump due to an mtrR mutation is required for chromosomally mediated penicillin resistance in Neisseria gonorrhoeae. *J Bacteriol* 184:5619-24

167. Virji M. 2009. Pathogenic neisseriae: surface modulation, pathogenesis and infection control. *Nat Rev Microbiol* 7:274-86

168. Walker CK, Sweet RL. 2011. Gonorrhea infection in women: prevalence, effects, screening, and management. *Int J Womens Health* 3:197-206

169. Walsh C. 2003. *Antibiotics : actions, origins, resistance*. Washington, D.C.: ASM Press. x, 335 p. pp.

170. Warner DM, Folster JP, Shafer WM, Jerse AE. 2007. Regulation of the MtrC-MtrD-MtrE efflux-pump system modulates the in vivo fitness of Neisseria gonorrhoeae. *The Journal of infectious diseases* 196:1804-12

171. Warner DM, Shafer WM, Jerse AE. 2008. Clinically relevant mutations that cause derepression of the Neisseria gonorrhoeae MtrC-MtrD-MtrE Efflux pump system confer different levels of antimicrobial resistance and in vivo fitness. *Molecular microbiology* 70:462-78

172. Wei Y, Guffanti AA, Ito M, Krulwich TA. 2000. Bacillus subtilis YqkI is a novel malic/Na+-lactate antiporter that enhances growth on malate at low protonmotive force. *J Biol Chem* 275:30287-92

173. Weyand NJ, Calton CM, Higashi DL, Kanack KJ, So M. 2010. Presenilin/gamma-secretase cleaves CD46 in response to Neisseria infection. *J Immunol* 184:694-701

174. Weyand NJ, Wertheimer AM, Hobbs TR, Sisko JL, Taku NA, et al. 2013. Neisseria infection of rhesus macaques as a model to study colonization, transmission, persistence, and horizontal gene transfer. *Proc Natl Acad Sci U S A* 110:3059-64

175. World Health Organization. 2014. *Antimicrobial resistance : global report on surveillance*. Geneva, Switzerland: World Health Organization. xxii, 232 pages pp.

176.   Wu H, Jerse AE. 2006. Alpha-2,3-sialyltransferase enhances Neisseria
        gonorrhoeae survival during experimental murine genital tract infection. *Infect
        Immun* 74:4094-103
177.   Yang QL, Gotschlich EC. 1996. Variation of gonococcal lipooligosaccharide
        structure is due to alterations in poly-G tracts in lgt genes encoding glycosyl
        transferases. *J Exp Med* 183:323-7
178.   Yokoi S, Deguchi T, Ozawa T, Yasuda M, Ito S, et al. 2007. Threat to cefixime
        treatment for gonorrhea. *Emerg Infect Dis* 13:1275-7
179.   Zhao S, Duncan M, Tomberg J, Davies C, Unemo M, Nicholas RA. 2009.
        Genetics of chromosomally mediated intermediate resistance to ceftriaxone and
        cefixime in Neisseria gonorrhoeae. *Antimicrob Agents Chemother* 53:3744-51